

79

# THE BOTANICAL GAZETTE

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EDITOR  
E. J. KRAUS

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VOLUME 101

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WITH FIVE HUNDRED AND SIXTY-TWO FIGURES



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## TABLE OF CONTENTS

	PAGE
Translocation of nitrogenous substances in the Cuthbert raspberry (with three figures) - -	<i>Charles J. Engard</i> 1
Anatomy of the aerial axes of <i>Equisetum kan-</i> <i>sanum</i> (with one figure) - - - - -	<i>Isabel M. P. Browne</i> 35
Chromosome numbers in root nodules and root tips of certain Leguminosae (with thirty-eight figures) - - - - -	<i>Louise Wipf</i> 51
Physiology of lazy corn (with three figures) -	<i>John Shafer, Jr.</i> 68
✓ Localization of photoperiodic perception in <i>Helianthus tuberosus</i> (with four figures) - -	<i>Karl C. Hamner and</i> <i>Elbert M. Long</i> 81 ✓
A plant growth inhibitor and plant growth in- hibition (with eleven figures) - - - -	<i>William S. Stewart</i> 91
Effects of soil temperature, pH, and nitrogen nutrition on the development of <i>Poa pratensis</i>	<i>Robert A. Darrow</i> 109
Leaf growth hormones. I. A bio-assay and source for leaf growth factors (with six figures) - -	<i>David M. Bonner,</i> <i>A. J. Haagen-Smit,</i> <i>and F. W. Went</i> 128
✓ Effect of variation in temperature during photo- periodic induction upon initiation of flower primordia in Biloxi soybean - - - -	<i>M. W. Parker and</i> <i>H. A. Borthwick</i> 145 ✓
✓ Photoperiodic induction as influenced by environ- mental factors (with two figures) - - -	<i>Elbert M. Long</i> 168 ✓
✓ Structure of the shoot apex in <i>Zamia</i> (with eleven figures) - - - - -	<i>Marion A. Johnson</i> 189
Histological and physiological responses of bean plants to alpha naphthalene acetamide (with fifteen figures) - - - - -	<i>E. J. Kraus and</i> <i>J. W. Mitchell</i> 204
Effectiveness of several growth substances on parthenocarp in holly - - - - -	<i>F. E. Gardner and</i> <i>Paul C. Marth</i> 226
✓ Nutrition of the myxomycetes. I. Pure culture and two-membered culture of myxomycete plasmodia (with four figures) - - - -	<i>Arthur L. Cohen</i> 243

Use of the Feulgen reaction in cytology. I. Effect of fixatives on the reaction (with three figures)	B. B. Hillary	276
Ontogenetic and anatomical studies of the flower and fruit of the Fagaceae and Juglandaceae (with seventy figures) - - - - -	LaDema M. Langdon	301
Conduction of rainfall by plant stems in a tropical rain forest (with two figures) - - - - -	Paul D. Voth	328
✓ Photoperiodic responses of several varieties of soybeans (with ten figures) - - - - -	H. A. Borthwick and M. W. Parker	341 ✓
✓ Effects of temperature, calcium, and arsenous acid on seedlings of <i>Poa pratensis</i> (with three figures) - - - - -	Aubrey W. Naylor	366
A morphological study of <i>Amplisiphonia</i> , a new member of the Rhodomelaceae (with thirteen figures) - - - - -	George J. Hollenberg	380
Stem anatomy of chaparral shrubs (with fourteen figures) - - - - -	Katherine S. Watkins	391
Chemical determination of ethylene in the emanations from apples and pears - - - - -	Elmer Hansen and Bert E. Christensen	403
Comparison of growth responses induced in plants by naphthalene acetamide and naphthalene acetic acid (with ten figures) - - - - -	John W. Mitchell and Wm. S. Stewart	410
✓ Growth substances and gametic reproduction in <i>Phycomyces</i> (with five figures) - - - - -	William J. Robbins	428
Evidence for auxin production in isolated roots growing in vitro - - - - -	J. van Overbeek	450
Vernalization of Marquis wheat and other spring cereals (with six figures) - - - - -	D. J. Wort	457 ✓
Chromosome number in some tulip hybrids -	Ronald Bamford, George B. Reynard, and John M. Bellows, Jr.	482
Further experiments on the relation of vitamin B <sub>1</sub> to the growth of green plants (with one figure)	James Bonner and Jesse Greene	491
Color and keeping quality of cut flowers - -	Marshall S. Neff	501
Layering in eastern white pine (with one figure)	H. J. Lutz	505

✓ Biochemical nitrogen fixation studies. I. Evidence for limited oxygen supply within the nodule (with six figures) - - - - -	F. E. Allison, C. A. Ludwig, Sam R. Hoover, and F. W. Minor	513
✓ Biochemical nitrogen fixation studies. II. Comparative respiration of nodules and roots, including non-legume roots (with two figures) -	F. E. Allison, C. A. Ludwig, F. W. Minor, and Sam R. Hoover	534
Cytological studies of <i>Lilium tigrinum</i> (with sixty-nine figures) - - - - -	Jonathan J. Westfall	550
Structure of fossil stem of Pityean affinity from the Reed Springs formation of Missouri (with nineteen figures) - - - - -	J. E. Cribbs	582
✓ Deterioration of grassland from stability to denudation with decrease in soil moisture (with seventeen figures) - - - - -	J. E. Weaver and F. W. Albertson	598
Size of nuclei in the shoot of <i>Ricinus communis</i> (with seven figures) - - - - -	Flora Murray Scott	625
✓ Growth responses of Biloxi soybeans to variation in relative concentrations of phosphate and nitrate in the nutrient solution (with four figures) - - - - -	Charles L. Hammer	637
✓ <i>Avena</i> coleoptile assay of ether extracts of nodules and roots of bean, soybean, and pea - - -	George K. K. Link and Virginia Eggers	650
✓ Interrelation of light and darkness in photo-periodic induction (with five figures) - - -	Karl C. Hammer	658 ✓
Effect of naphthalene acetic acid and naphthalene acetamide on nitrogenous and carbohydrate constituents of bean plants (with two figures)	John W. Mitchell	688
✓ Interrelations in the effects of boron and indoleacetic acid on plant growth (with one figure)	Frank M. Eaton	700
Light stability of auxin in <i>Avena</i> coleoptiles -	W. S. Stewart and F. W. Went	706
Permanent changes of chloroplasts induced by X rays in the gametophyte of <i>Polypodium aureum</i> (with forty-six figures) - - - - -	Lewis Knudson	721
Spore germination and vegetative stages of the gametophytes of <i>Hymenophyllum</i> and <i>Trichomanes</i> (with thirty-five figures) - - -	Alma G. Stokey	759

Absorption of soil moisture by maize roots (with three figures) - - - - -	<i>Charles Homer Davis</i>	791
✓ Floral initiation in Biloxi soybeans as influenced by age and position of leaf receiving photo-periodic treatment - - - - -	<i>H. A. Borthwick and M. W. Parker</i>	806
Growth and development of the embryo and fruit of the peach as affected by ringing and defoliation of the branches (with four figures) - -	<i>H. B. Tukey and F. A. Lee</i>	818
✓ Cytological investigations in <i>Desmodium</i> and <i>Lespedeza</i> (with forty-three figures) - -	<i>J. Oran Young</i>	839
Sex expression in willows (with four figures) -	<i>Ernest C. Smith</i>	851
Density of stomata and oil glands and incidence of water spot in the rind of Washington navel orange (with nine figures) - - - - -	<i>F. M. Turrell and L. J. Klotz</i>	862
Origin of adventitious shoots in decapitated cranberry seedlings (with fifteen figures) -	<i>Henry F. Bain</i>	872
Effect of naphthalene acetic acid on mobile auxin in bean seedlings (with one figure) - - -	<i>William S. Stewart</i>	881
Growth of incompatible pollen tubes in <i>Oenothera organensis</i> (with six figures) - - - - -	<i>Sterling Emerson</i>	890
✓ Effect of potato extracts on growth of <i>Phycomyces</i> (with three figures) - - - - -	<i>William J. Robbins and Karl C. Hamner</i>	912
<i>Avena</i> coleoptile assay of ether extracts of aphids and their hosts - - - - -	<i>George K. K. Link, Virginia Eggers, and James E. Moulton</i>	928
Auxin in marine plants. II - - - - -	<i>J. van Overbeek</i>	940
Morphological study of <i>Exormotheca tuberifera</i> (with twenty-five figures) - - - - -	<i>Sultan Ahmad</i>	948
BRIEFER ARTICLES—		
ADOLF CARL NOE, obituary notice - - -	<i>E. J. Kraus</i>	231
Polyembryony in <i>Myrciaria cauliflora</i> - -	<i>H. P. Traub</i>	233
HENRY CHANDLER COWLES, obituary notice -	<i>E. J. Kraus</i>	241
Fluorescent lamps as a source of light for growing plants - - - - -	<i>A. W. Naylor and Gladys Gerner</i>	715
CURRENT LITERATURE - - - - -		235, 508, 717, 955
For titles of book reviews see index under author's name		

# THE BOTANICAL GAZETTE

September 1939

## TRANSLOCATION OF NITROGENOUS SUBSTANCES IN THE CUTHBERT RASPBERRY<sup>1</sup>

CHARLES J. ENGARD

(WITH THREE FIGURES)

### Introduction

In studies dealing with the translocation of nitrogenous compounds in plants, there seems to have been a tendency to search for the same plan of movement which has been observed for carbohydrates. It is apparent, however, that much of the translocation of nitrogen will differ markedly from that of carbohydrates, because the places of syntheses, unlike photosynthesis, may be found in almost any or all parts of the plant (6, 18, 16, 17, 19, 20, 21, 8).

This paper presents the results of a study of the translocation and metabolism of the nitrogenous substances of the Cuthbert raspberry, and correlates these with the results of a study of carbohydrates obtained at the same time and already reported (7).

Recent literature dealing with nitrogen metabolism of plants has been reviewed by NIGHTINGALE (15) and MCKEE (13). The translocation of nitrogenous substances has been discussed by CLEMENTS (1), CURTIS (4), and MASON and PHILLIS (12).

### Material and methods

The Cuthbert raspberry, a hybrid of *Rubus strigosus* × *R. idaeus*, each year produces tall, erect, biennial, vegetative canes from peren-

<sup>1</sup> Contribution no. 58 from the botany department of the State College of Washington.

nial roots. These vegetative canes develop all of their height and most of their diameter during the first season, and in the second season bear fruit on short lateral branches. The studies here reported refer only to the first year or vegetative plants.

**TREATMENT AND SAMPLING.**—Double rings were used to check movements of materials in the bark, one 15 cm. and another 30 cm. above the ground, thereby isolating a 15 cm. segment of bark between the rings. For analysis the canes were cut at the rings and the portion of the cane above the rings was cut into 15 cm. lengths. The control plants likewise were cut for analysis into 15 cm. lengths, measuring from the ground upward; the second segment of the control thus corresponds with the isolated segment (between the rings) of the treated plant.

Canes were selected in three groups of twelve plants each. The canes of the first group were removed by cutting at the ground level, and these served as controls for the beginning of the experiment. The stems of the second group were ringed in the manner described. The canes of the third group were tagged (but not ringed) and left to be removed as controls at the time of the next ringing treatment. The first treatment and collection of controls were made on May 15. The ringed plants and the tagged plants were harvested on June 23. Thus the tagged plants served as end controls for the May 15 series and as beginning controls for the June 23 series. More canes were ringed and others tagged to be left as controls on June 23. These were harvested on July 15, and the procedure repeated. The plants ringed and those tagged for end controls on July 15 were collected on August 9. These were the end controls for the whole seasonal study.

**MOISTURE.**—The moisture content of the canes employed was assumed to be the same as that obtained for the similar canes used in the carbohydrate studies.

**NITROGEN DETERMINATION.**<sup>2</sup>—The fresh plant material, which had been autoclaved at 15 lb. pressure for 15 minutes, was ground in a plate grinder with the addition of generous quantities of water. This mixture was brought to a boil, and filtered with suction after

<sup>2</sup> Unless otherwise specified, the term nitrogen is used in this paper as an abbreviation of the term nitrogenous compounds.

the addition of 10 cc. of 10 per cent acetic acid to coagulate the colloidal nitrogen. The residue after several washings was discarded. The filtrate was diluted to a volume of 2 liters, and this was the stock solution from which aliquots were taken for determination of the various fractions. The procedure of fractionation was that reported by LEONARD (8).

EXPRESSION OF RESULTS.—The percentages of nitrogen, as well as the absolute amounts, are given for each segment of stem, and for the leaves of the segment. The latter were analyzed separately from the stem segment. For convenience in reporting the results, the segments of stem were designated, from the ground upward, as 1st, 2st, 3st, etc.; the corresponding leaves were designated as 1L, 2L, 3L, etc. Thus in the ringed plants 1st is that segment of stem below the lower ring, 2st is that between the rings, and 3st is that just above the upper ring.

### Investigation

#### DISTRIBUTION OF NITROGENOUS SUBSTANCES IN NORMAL PLANTS, AND EFFECT OF RINGING

TOTAL ORGANIC NITROGEN.—Within the cane the highest percentages of total organic nitrogen are found in the topmost stem segment and leaves, with a downward gradient in the stem to the lowest concentration in the basal segment. This might be expected in view of the meristematic tissues present in the tip in contrast to the senescent tissues at the base. The percentage figures of total organic nitrogen are little more than indices of the proportion of living, protoplasm-containing cells (meristems, cambiums, parenchyma) to non-living, non-protoplasmic cells with thickened walls (tracheids, vessels, sclerenchyma). In general, as the plants age with the progress of summer, cell wall and secondary thickening continue, residual carbohydrates increase, while a considerable portion of the protoplasm disappears and the percentages of total organic nitrogen decrease (tables 1, 2; figs. 1, 2).

In all segments of the stem above the lower ring, including the isolated segment, the loss of nitrogen (percentage of residual dry weight) is not so great as in the normal stems; that is, there is more organic nitrogen per gram of residual dry weight left at the end of

the period of treatment in the ringed than in the normal stems. Below the lower ring, in the basal segment, loss of organic nitrogen is greater than normal. These results are caused by the accumulation of carbohydrates and other substances above the ring, which are effective in producing callus growth as well as accelerated growth in diameter of the region above the rings. The increased growth means in turn increased amounts of protoplasm, with its abundance of nitrogen. Relative to the residual polysaccharides in the stem,

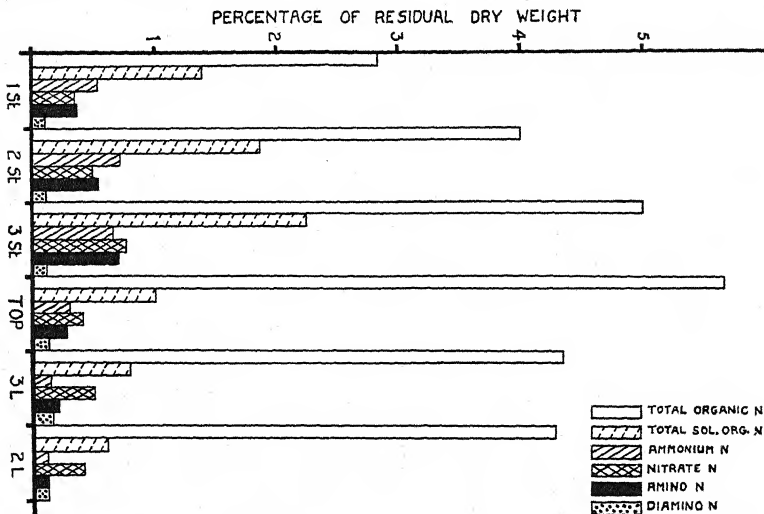


FIG. 1.—Distribution of nitrogenous substances in normal plants; May 15

the percentage of the latter is greater. Thus ringing results in a higher organic nitrogen content of the stem immediately above the rings by increasing the amount of living tissue, but this effect is manifested by a smaller percentage loss of nitrogen above the rings as the summer progresses. There is a loss of total organic nitrogen below the rings, owing to the apparent prevention of growth of the basal stem segment. The effect of the rings in preventing the downward movement of carbohydrates may be partly responsible for the inhibited growth; but since carbohydrate does move into the basal segment from the roots (7), the effect may be more directly a result of the prevention of downward movement of growth regulators.

The leaves of the June plants developed, with the exception of those growing on segment 3st, after the rings were made on May 15.

The organic nitrogen content of these leaves was much lower than that of the leaves which developed on the normal plants during the

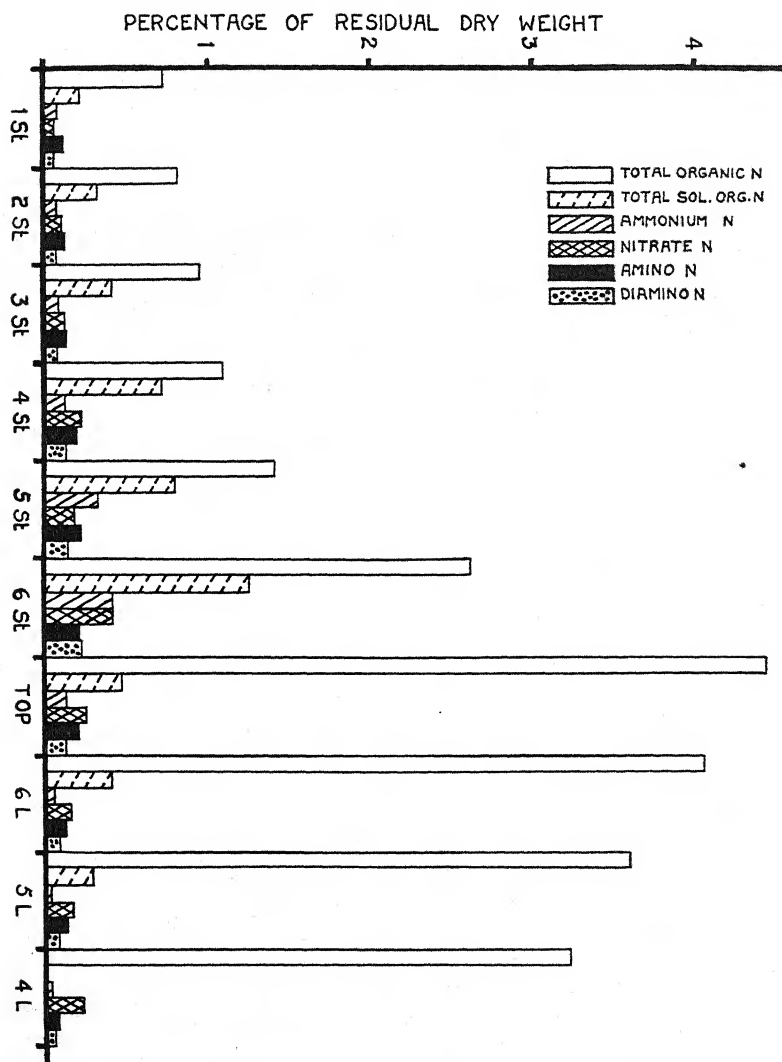


FIG. 2.—Distribution of nitrogenous substances in normal plants; June 23

same time, indicating that ringing had in some way inhibited the normal formation of protein in the leaves, possibly by disruption of water relations. The leaves of the plants ringed on June 23 and

gathered on July 16 showed considerable reduction in percentage of organic nitrogen, whereas the normal plants showed an increase in percentage of total organic nitrogen. The leaves of the treated plants of the August collection, however, showed substantial increases over the normal plants. These were ringed on July 16, when the plants were mature, and the treatment apparently caused less injury than it did on the plants collected in June and July.

When the plants were ringed on May 15 they consisted of three segments and the tops; that is, they were approximately 50 cm. tall. When they were harvested on June 23 they were six segments (plus tops) tall, about 100 cm. By June 23 they had made approximately twice the growth in height, while the dry weight of the stem above the upper rings increased from 20.9 gm. at the time of ringing on May 15 to 72.4 gm. by June 23. The dry weight of the corresponding portion of the stem of the normal plants increased from 20.9 gm. on May 15 to 63.5 gm. on June 23. The plants harvested on July 16 had not increased so greatly in height over their beginning controls (June 23 normal canes) as had those of June. Although data for the uppermost segment and tops are not available, a comparison of the remaining segments of the normal plants of July 16 with those of June 23 reveals that there was a small loss of organic nitrogen per segment in grams and in percentage of residual dry weight, whereas from May 15 to June 23 there was considerable loss from each segment. The ringed canes collected on July 16 showed a slight increase in organic nitrogen over the beginning controls collected on June 23. Similar relationships obtained in the August plants.

Three factors are operative in causing a decrease in nitrogen per segment in the normal and in the ringed plants. The first of these factors is essentially a "dilution," brought about by increasing cell wall thickening and by the production of secondary tissue in which thick-walled xylem is most abundant. The second factor is the large amount of secondary xylem produced in May and June when differentiation of vessels and tracheids results in a loss of protoplasm. The nitrogenous constituents of the protoplasm, not entering into secondary wall formation, perhaps move to the rapidly growing upper portion of the plant. The third factor concerns the loss of

nitrogen from the stem, either by migration to the roots or directly to the environment.

Ringling interferes with the carbohydrate status of the stem by causing a large accumulation above the rings. Where carbohydrates accumulate so also do nitrogenous compounds if inorganic nitrogen is available. There appears to be considerable synthesis of organic nitrogen throughout the whole length of the stem, since growth continues and is usually stimulated by the abundance of carbohydrates. Thus above the rings the dilution factor is even more important than it is in unringed canes. Accumulation of nitrogen by a carbohydrate-gorged stem, and an increased amount of protoplasm owing to accelerated growth, operate against the loss of nitrogenous substances by dilution through cell wall thickening and loss of protoplasm incidental to the differentiation of tracheae, tracheids, and fibers. From May 15 to June 23 percentage loss of organic nitrogen in the ringed plants was greater than accumulation, but the net loss was smaller than that of normal plants during the same period. From June 23 to July 16, and from July 16 to August 9, loss in percentage of organic nitrogen in the ringed segments was less than the accumulation of nitrogen, owing to the abundance of carbohydrates; and the organic nitrogen concentration was greater than that of the beginning controls of each group. The normal plants also lost organic nitrogen through the factors previously mentioned. Thus when dealing with amount of organic nitrogen per unit of any maturing or differentiating tissue, the normal process is to lose organic nitrogen; when accumulations result through any means in a maturing or differentiating tissue, the accumulation is a net result which includes loss through thickening and differentiation. In this paper the term accumulation is used in that sense, and its criterion is a concentration higher than that of the normal plants harvested at the same time. For example, in the ringed plants harvested in June the concentration of organic nitrogen is higher than that of the normal plants taken at the same time, but the concentration in each is lower than that of its beginning control, the normal plants of May 15.

**SOLUBLE ORGANIC NITROGEN AND PROTEIN.**—Concentration of the soluble forms of organic nitrogen increases in the stem from the

basal to the subterminal segment, where occurs the highest concentration of all regions of the entire plant (tables 1, 2; figs. 1, 2). Concentration of this composite fraction in the tops and in the leaves is more than 50 per cent lower than in the subterminal stem segment. Since the concentration of total organic nitrogen was highest in the tops (the portion including the stem tip and small unfolding leaves), it appears that concentration of the protein fraction, obtained by subtracting the soluble nitrogen from the total organic nitrogen,<sup>3</sup> was highest in the stem tip and small leaves where the tissues are little differentiated and mostly parenchymatous. The young leaves are largely meristematic, and the amount of protoplasm per unit of residual dry weight is higher in this than in any other region of the plant. Thus the content of soluble nitrogen is low in the leaves and stem tip, while the protein content is high. This seems to indicate rapid utilization of the soluble forms in the synthesis of protein in this region of great meristematic activity.

Soluble nitrogen, like the total organic, showed a gradual decrease in concentration per segment with the progress of summer, brought about by the factors already discussed. A similar decrease in soluble nitrogen from an early season high was noted by LEONARD (8) in the sunflower.

Below the lower ring in the treated plants there was an accumulation of soluble organic nitrogen which was twice that of the corresponding segments of the control. In the treated plants of July the accumulation not only doubled the amount of soluble organic nitrogen in the check plants taken at the same time, but was greater than that of the beginning controls harvested on June 23. There was no such accumulation in the more mature August plants.

Between the rings the percentage of soluble organic nitrogen was above the normal (fig. 3). The value for this segment is normally intermediate between that of the first and third segments in the untreated plants. The total organic nitrogen, which includes protein, is intermediate in concentration in the segment between the rings. This indicates that proteolysis occurred to some extent, increasing the amounts of the soluble forms, particularly alpha amino acids

<sup>3</sup> The figures for protein are at best only rough estimates, and should be interpreted accordingly.

and ammonium nitrogen, with a corresponding loss of protein (fig. 3). A similar accumulation of nitrogen appeared in the ringed plants of the July and August collections.

Above the rings the concentration of soluble organic forms remained approximately normal in all series. The concentrations in the leaves of the ringed plants collected in June were lower than normal, possibly owing to inhibition of the growth rate by ringing so early in the life of the plant. The fact that there was a larger amount of soluble organic nitrogen in the stem above the rings than in the normal stem is significant, because when coupled with the increase of total organic nitrogen above normal it means that protein synthesis continued beyond the normal rate, owing to an abundance of carbohydrates. Apparently reduction of nitrates and their change to amino acids and protein take place all along the stem, the amount of reduction being proportional to the number of living cells per unit of tissue. The nitrogen supply to this region is continuous and therefore must come from the xylem. The rings do not seriously interfere with this nitrogen supply, although accumulation of the soluble fraction below the lower ring in the June and July collections suggests interference with upward movement from the roots.

The total organic nitrogen concentration was lower than normal in the basal segment of the ringed plants collected in June and July, but the total soluble forms were higher, indicating that although

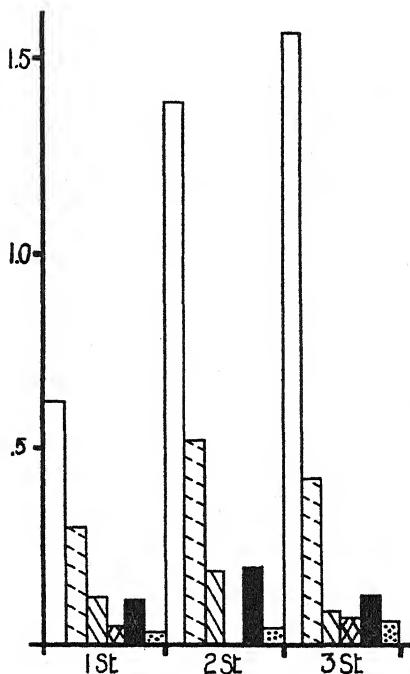


FIG. 3.—Effect of ringing on nitrogen fractions in first three segments of stem; June 23.

soluble forms accumulated below the ring, the cessation of diameter growth and partial proteolysis resulted in a loss of protein which itself probably contributed to the accumulation of soluble forms. Ringing the mature plant in July did not affect the concentration of total organic and total soluble organic nitrogen fractions in the same way, because there was an accumulation of total organic and a decrease in soluble organic nitrogen in the basal segment. This points to the likelihood of proteolysis contributing to the accumulation of soluble nitrogen rather than to interference with upward movement. The data indicate the existence of a reversible equilibrium between the soluble organic and the insoluble (protein) nitrogen.

ALPHA AMINO ACIDS.—The soluble organic fractions which are present in significant quantities are the alpha amino acids and the diamino (basic) nitrogenous compounds. Of these the alpha amino acids are more important, judging from their reactions to treatment and their closer relationship to the total soluble fraction and the proteins.

The concentration of this fraction was higher in the beginning control plants collected May 15 than in the plants of any other collection. There was a steady though not rapid decline in concentrations of alpha amino acids to the end of the season. The large concentrations in May were present during the period of most rapid growth,<sup>4</sup> when protein formation necessitates rapid reduction of nitrate and its ultimate transformation to amino acids. The steady loss in amounts of amino acids throughout the summer was produced not only by the factors of dilution, loss of protoplasm, and direct loss of nitrogen to the roots or to the environment, but also by a declining absorption of nitrate from soil whose nitrate supply is normally diminished by the middle of summer. In the August series the alpha amino and diamino acids constituted most of the total soluble nitrogen.

Within the normal plant the concentrations of alpha amino acids increased from the basal to the subterminal segment, in which was found the largest amount of alpha amino acids in the plant. This

<sup>4</sup> During the 5½ weeks between the May 15 and June 23 collections, the canes grew about 50 cm. in length ("tops" usually not 15 cm. in length).

fraction was abundant in the May stems (table 1; fig. 1), and was a prominent contributor to the soluble nitrogen peak in the subterminal segment. The concentration of alpha amino acids in the stem tip part-segment was more than 50 per cent lower than that of the subterminal segment. The leaves were 50 per cent lower in alpha amino acid content than their corresponding stem segments. As the season progressed the stem and leaf concentrations approached each other more closely.

It was pointed out previously that where concentration of the protein fraction was greatest, total soluble fraction was lowest (stem tip); and that just below the stem tip, in the subterminal segment, concentration of the soluble forms was considerably greater, with protein correspondingly lower, than it was in the stem tip. Since alpha amino acids contribute most to this difference in concentration of soluble forms, the same relationships hold.

In the ringed plants of the June and July collections there was an accumulation of alpha amino nitrogen in the basal segment, in the segment (2st) between the rings, and in the segment (3st) just above the rings (table 4). The concentration of this fraction in the ringed plants of the August collection remained nearly normal in segments 1st and 3st, but there was considerably more alpha amino nitrogen between the rings. Inhibiting the upward movement of amino acids from the roots or the downward movement from the upper regions might have produced the accumulation above and below the ring, but this could not account for the accumulation between the rings. Figure 3 shows, without reference to the controls, the distribution of total organic nitrogen and the fractions in the first three segments of the ringed canes of June. It is seen that the total organic nitrogen, which increased only slightly over the control segment, is on the graph nearly in its normal position; that is, it is intermediate between that of the first and that of the third segment. The total soluble fraction, however, increased well beyond its intermediate position, and consequently protein decreased. The increase in total soluble nitrogen, including mostly an increase in alpha amino acids, occurred at the expense of the protein. Thus it seems that the accumulation of alpha amino acids in the segment

between the rings was a result of proteolysis, and this is borne out by the external appearance of this isolated segment.

The segment above the ring had an abundance of sugars, and was actually stimulated to greater diameter growth. Considerable callus rapidly developed, with the incidental widespread meristematic activity. Synthesis of amino acids progressed at a rapid rate and there was a great increase in total organic nitrogen, much of which was protein. The accumulation above the ring therefore appeared to be produced by the synthesis of amino acids and protein, rather than by a stopping of downward movement.

The accumulation of alpha amino acids below the rings is partly reflected in the accumulation of the total soluble fraction. The total organic nitrogen value was lower than normal, and this (with the accumulation of soluble forms) points to loss of protein. It is possible that some amino acids moved in from the roots. This segment, not so senescent as that between the rings, did not increase in diameter; in fact, its cambial activity is normally very weak. A small amount of sugar moved into this segment from the roots, especially in the spring (7), and the segment lives even though the important influx of carbohydrates from above has been prevented by ringing. Ringing later in the life of the plant had no such effect on the basal segment.

**DIAMINO ACIDS.**—The diamino or basic nitrogen fraction contains such acids as proline, lysine, etc. The concentrations in the stems increase very gradually up to an amount equal to and often larger than the alpha amino acids in the plants of the August collection. The quantities of diamino acids in the leaves of all plants are small and often negligible (tables 1, 4).

In the stems and leaves above the rings the concentration of this fraction was slightly lower than normal. Like the alpha amino, the diamino acids accumulated in small amounts below the rings in the plants of the June collection, but there was no accumulation below the rings in the plants collected in July. In the treated plants of the August collection the concentration of this fraction was lower than normal. There were no significant differences between the concentrations in the second segment of the normal and ringed plants.

The concentrations of diamino acids seem to have some relationship to those of alpha amino acids.

AMIDES.—The concentrations of the amide fraction were insignificant (tables 1, 5).

TABLE 1  
DISTRIBUTION OF NITROGENOUS SUBSTANCES IN MAY 15 CONTROLS

CANE SEGMENT	Gm.	PERCENT- AGE RESIDU- AL DRY WEIGHT	Gm.	PERCENT- AGE RESIDU- AL DRY WEIGHT	Gm.	PERCENT- AGE RESIDU- AL DRY WEIGHT	Gm.	PERCENT- AGE RESIDU- AL DRY WEIGHT
	TOTAL ORGANIC NITROGEN		SOLUBLE ORGANIC NITROGEN		AMMONIUM NITROGEN		NITRATE NITROGEN	
1st.....	0.665	2.82	0.3280	1.391	0.1224	0.520	0.0826	0.350
2st.....	0.645	3.99	0.3020	1.855	0.1164	0.716	0.0781	0.488
3st.....	0.415	5.07	0.1840	2.253	0.0540	0.662	0.0620	0.760
Top.....	0.424	5.68	0.0740	0.988	0.0208	0.279	0.0288	0.386
3L.....	1.500	4.37	0.2720	0.792	0.0432	0.126	0.0864	0.252
2L.....	1.461	4.30	.....	.....	0.0400	0.118	0.1300	0.383
	ALPHA AMINO NITROGEN		DIAMINO NITROGEN		AMIDE NITROGEN			
1st.....	0.0858	0.364	0.0240	0.102	0.0000	0.000		
2st.....	0.0844	0.519	0.0168	0.103	0.0042	0.026		
3st.....	0.0583	0.715	0.0092	0.112	0.0023	0.028		
Top.....	0.0199	0.267	0.0091	0.122	0.0008	0.011		
3L.....	0.0610	0.178	0.0512	0.149	0.0025	0.007		
2L.....	0.0425	0.125	0.0400	0.118	0.0014	0.006		

NITRATES.—This fraction, which includes nitrates and nitrites, was abundant throughout the entire plant early in the season (tables 1, 3). Its concentration in the leaves and stem tip of the May, June, and July series was nearly as high as in the stems. The concentration was very low in stem and leaves of the plants collected in August, but nitrates were supplied to the tops of the plants, for ammonium nitrogen was always present, together with amino acids, even when nitrates were very low. Continuous reduction utilizes much of the nitrate reaching the top of the plant. There is no doubt that nitrates were absorbed in greater quantities in the

TABLE 2

DISTRIBUTION OF TOTAL ORGANIC AND TOTAL SOLUBLE ORGANIC NITROGEN IN NORMAL AND RINGED CANES

FRACTION	CANE SEGMENT	JUNE 23				JULY 16				AUGUST 9			
		CONTROLS		RINGED		CONTROLS		RINGED		CONTROLS		RINGED	
		Gm.	PERCENT-AGE RESIDUAL DRY WEIGHT	Gm.	PERCENT-AGE CONTROL RESIDUAL DRY WEIGHT	Gm.	PERCENT-AGE RESIDUAL DRY WEIGHT	Gm.	PERCENT-AGE CONTROL RESIDUAL DRY WEIGHT	Gm.	PERCENT-AGE RESIDUAL DRY WEIGHT	Gm.	PERCENT-AGE CONTROL RESIDUAL DRY WEIGHT
Total organic nitrogen	1st. ....	0.1590	0.707	0.1390	0.618	0.2420	1.045	0.1255	0.541	0.1349	0.589	0.1925	0.834
	2st. ....	0.1300	0.807	0.2240	1.390	0.1293	0.746	0.1355	0.784	0.1210	0.673	0.1785	0.992
	3st. ....	0.1295	0.933	0.2180	1.570	0.1293	0.890	0.1440	1.000	0.1216	0.746	0.2010	1.234
	4st. ....	0.1165	1.068	0.2150	1.973	0.1040	1.067	0.1790	1.846	0.1000	0.765	0.1774	1.363
	5st. ....	0.1160	1.400	0.1445	1.810	0.1041	1.300	0.1015	1.270	0.0804	0.811	0.1198	1.222
	6st. ....	0.1075	2.615	0.1152	2.810	.....	.....	.....	.....	0.1688	0.885	0.1300	1.668
	7st. ....	.....	.....	.....	.....	.....	.....	.....	.....	0.0590	0.944	0.1180	1.870
	Top. ....	0.5070	4.400	0.4580	3.550	.....	.....	.....	.....	0.5100	3.300	0.4380	2.880
	7L. ....	.....	.....	.....	.....	.....	.....	.....	.....	0.4770	2.935	0.6050	3.710
	6L. ....	0.6790	4.020	0.4840	2.865	.....	.....	.....	.....	0.4880	2.800	0.5030	3.230
Total organic nitrogen	5L. ....	0.7280	3.570	0.4910	2.410	0.6540	3.270	0.3210	1.603	0.4260	.....	0.2940	.....
	4L. ....	0.6130	3.210	0.4600	2.425	0.4930	2.762	0.2175	1.222	.....	.....	.....	.....
	3L. ....	0.4280	2.580	0.4070	2.455	0.4825	2.360	.....	.....	.....	.....	.....	.....
	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....

TABLE 2—Continued

FRACTION	CANE SEGMENT	JUNE 23				JULY 16				AUGUST 9			
		CONTROLS		RINGED		CONTROLS		RINGED		CONTROLS		RINGED	
		Gm.	PERCENT-AGE RESIDUAL DRY WEIGHT	Gm.	PERCENT-AGE CONTROL RESIDUAL DRY WEIGHT	Gm.	PERCENT-AGE RESIDUAL DRY WEIGHT	Gm.	PERCENT-AGE CONTROL RESIDUAL DRY WEIGHT	Gm.	PERCENT-AGE RESIDUAL DRY WEIGHT	Gm.	PERCENT-AGE CONTROL RESIDUAL DRY WEIGHT
Total soluble organic nitrogen	1st. ....	0.0397	0.176	0.0768	0.342	0.0257	0.111	0.0557	0.240	0.0660	0.289	0.0448	0.105
	2st. ....	0.0576	0.356	0.0840	0.522	.....	.....	0.0768	0.444	0.0510	0.284	0.0640	0.350
	3st. ....	0.0525	0.378	0.0548	0.420	0.0472	0.328	0.0383	0.266	0.0447	0.274	0.0384	0.236
	4st. ....	0.0749	0.684	0.0608	0.553	0.0597	0.616	.....	.....	0.0510	0.390	0.0448	0.344
	5st. ....	0.0640	0.800	.....	.....	0.0597	0.746	0.0426	0.533	0.0480	0.487	0.0256	0.261
	6st. ....	0.0512	1.248	0.0450	1.100	.....	.....	.....	.....	0.0447	0.574	0.0384	0.493
	7st. ....	.....	.....	.....	.....	.....	.....	.....	.....	0.0447	0.709	0.0725	1.150
	Top. ....	0.0832	0.453	0.0650	0.504	.....	.....	.....	.....	0.0704	0.463	0.0575	0.378
	7L. ....	.....	.....	.....	.....	.....	.....	.....	.....	0.0576	0.353	0.0575	0.353
	6L. ....	0.0640	0.379	0.0450	0.266	.....	.....	.....	.....	0.0545	0.334	0.0640	0.368
	5L. ....	0.0768	0.377	0.0480	0.235	0.0425	0.214	0.0423	0.211	0.0576	.....	0.0348	.....
	4L. ....	.....	.....	0.0608	0.319	0.0409	0.264	.....	.....	0.0545	0.216	.....	.....
	3L. ....	0.0576	0.347	0.0400	0.242	0.0512	0.251	.....	.....	.....	.....	.....	.....

TABLE 3  
DISTRIBUTION OF ALPHA AMINO AND DIAMINO NITROGEN IN NORMAL AND RINGED CANES

FRACTION	CANE SEGMENT	JUNE 23				JULY 16				AUGUST 9			
		CONTROLS		RINGED		CONTROLS		RINGED		CONTROLS		RINGED	
		Gm.	PERCENT-AGE RESIDUAL DRY WEIGHT	Gm.	PERCENT-AGE CONTROL RESIDUAL DRY WEIGHT	Gm.	PERCENT-AGE RESIDUAL DRY WEIGHT	Gm.	PERCENT-AGE CONTROL RESIDUAL DRY WEIGHT	Gm.	PERCENT-AGE RESIDUAL DRY WEIGHT	Gm.	PERCENT-AGE CONTROL RESIDUAL DRY WEIGHT
Alpha amino nitrogen	1st.....	0.0099	0.088	0.0203	0.090	0.0046	0.020	0.0083	0.036	0.0181	0.079	0.0166	0.073
	2st.....	0.0152	0.094	0.0318	0.197	0.0079	0.045	0.0134	0.078	0.0171	0.095	0.0266	0.148
	3st.....	0.0137	0.098	0.0171	0.123	0.0104	0.072	0.0106	0.074	0.0146	0.090	0.0146	0.089
	4st.....	0.0186	0.104	0.0092	0.084	0.0111	0.114	0.0090	0.094	0.0146	0.111	0.0120	0.093
	5st.....	0.0164	0.206	0.0081	0.101	0.0124	0.155	0.0039	0.048	0.0165	0.168	0.0070	0.072
	6st.....	0.0076	0.187	0.0193	0.472	.....	.....	.....	.....	.....	.....	0.0055	0.071
	7st.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
	Top....	0.0209	0.162	0.0247	0.191	0.0184	.....	0.0214	.....	0.0086	0.137	0.0306	0.135
	7L.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
	6L.....	0.0170	0.100	0.0231	0.136	0.0092	.....	.....	.....	0.0196	0.125	0.0156	0.096
	5L.....	0.0211	0.103	0.0212	0.104	0.0164	0.082	0.0139	0.069	0.0201	0.113	0.0105	0.061
	4L.....	0.0104	0.054	0.0130	0.068	0.0145	0.081	0.0130	0.074	0.0316	.....	.....	.....
	3L.....	0.0118	0.071	0.0124	0.074	0.0099	0.049	.....	.....	0.0266	0.106	.....	.....

TABLE 3—Continued

FRACTION	CANE SEGMENT	JUNE 23				JULY 16				AUGUST 9			
		CONTROLS		RINGED		CONTROLS		RINGED		CONTROLS		RINGED	
		Gm.	PERCENT- AGE RESIDUAL DRY WEIGHT	Gm.	PERCENT- AGE CONTROL RESIDUAL DRY WEIGHT	Gm.	PERCENT- AGE RESIDUAL DRY WEIGHT	Gm.	PERCENT- AGE CONTROL RESIDUAL DRY WEIGHT	Gm.	PERCENT- AGE RESIDUAL DRY WEIGHT	Gm.	PERCENT- AGE CONTROL RESIDUAL DRY WEIGHT
Diamino nitrogen	1st.....	0.0048	0.022	0.0065	0.058	0.0085	0.037	0.0086	0.037	0.0176	0.077	0.0048	0.029
	2st.....	0.0067	0.041	0.0060	0.037	0.0107	0.062	0.0106	0.061	0.0192	0.106	0.0120	0.067
	3st.....	0.0051	0.036	0.0084	0.060	0.0085	0.059	0.0074	0.051	0.0160	0.098	0.0096	0.059
	4st.....	0.0115	0.105	0.0089	0.081	0.0107	0.109	0.0148	0.153	0.0144	0.110	0.0096	0.074
	5st.....	0.0088	0.110	0.0074	0.092	0.0171	0.213	0.0149	0.186	0.0160	0.102	0.0032	0.033
	6st.....	0.0080	0.196	0.0080	0.195	.....	.....	.....	.....	0.0160	0.206	0.0144	0.185
	7st.....	.....	.....	.....	.....	.....	.....	.....	.....	0.0160	0.254	0.0181	0.287
	Top.....	0.0128	0.099	0.0048	0.037	0.0075	.....	0.0130	.....	0.0192	0.126	0.0128	0.084
	7L.....	.....	.....	.....	.....	.....	.....	.....	.....	0.0160	0.098	0.0128	0.079
	6L.....	0.0096	0.057	0.0072	0.042	0.0085	.....	.....	.....	0.0128	0.072	0.0112	0.064
	5L.....	0.0112	0.055	0.0056	0.027	0.0107	0.054	0.0106	0.053	0.0128	.....	0.0112	.....
	4L.....	0.0088	0.046	0.0112	0.058	0.0107	0.060	0.0107	0.060	0.0144	0.057	.....	.....
	3L.....	0.0160	0.096	0.0056	0.033	0.0064	0.031	.....	.....	.....	.....	.....	.....

TABLE 4  
DISTRIBUTION OF AMMONIUM AND NITRATE ( $+NO_3$ ) NITROGEN IN NORMAL AND RINGED CANES

FRACTION	CANE SEGMENT	JUNE 23				JULY 16				AUGUST 9			
		CONTROLS		RINGED		CONTROLS		RINGED		CONTROLS		RINGED	
		Gm.	PERCENT-AGE RESIDUAL DRY WEIGHT	Gm.	PERCENT-AGE CONTROL RESIDUAL DRY WEIGHT	Gm.	PERCENT-AGE RESIDUAL DRY WEIGHT	Gm.	PERCENT-AGE CONTROL RESIDUAL DRY WEIGHT	Gm.	PERCENT-AGE RESIDUAL DRY WEIGHT	Gm.	PERCENT-AGE CONTROL RESIDUAL DRY WEIGHT
Ammonium nitrogen	1st.....	0.0096	0.042	0.0276	0.122	.....	.....	0.0236	0.101	0.0064	0.022	0.0043	0.018
	2st.....	0.0091	0.036	0.0300	0.186	.....	.....	0.0265	0.153	0.0032	0.017	0.0182	0.101
	3st.....	0.0088	0.063	0.0113	0.081	0.0106	0.073	0.0021	0.014	0.0048	0.029	0.0023	0.014
	4st.....	0.0107	0.098	0.0104	0.095	0.0064	0.065	0.0096	0.099	0.0032	0.024	0.0048	0.036
	5st.....	0.0235	0.291	0.0074	0.092	0.0043	0.053	0.0055	0.068	0.0016	0.016	0.0032	0.032
	6st.....	0.0160	0.393	0.0080	0.195	.....	.....	.....	.....	0.0032	0.041	0.0040	0.051
	7st.....	.....	.....	.....	.....	.....	.....	.....	.....	0.0048	0.077	0.0207	0.032
	Top ....	0.0128	0.099	0.0089	0.069	.....	.....	.....	.....	0.0064	.....	0.0032	0.021
	7L.....	.....	.....	.....	.....	.....	.....	.....	.....	0.0056	0.031	0.0056	0.034
	6L.....	0.0040	0.023	0.0050	0.029	.....	.....	.....	.....	0.0056	.....	0.0051	0.029
	5L.....	0.0056	0.027	0.0048	0.023	0.0000	0.000	0.0000	0.000	0.0064	0.025	0.0040	.....
	4L.....	0.0032	0.016	0.0024	0.012	0.0021	0.010	0.0009	0.005	.....	.....	.....	.....
	3L.....	0.0043	0.025	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....

TABLE 4—Continued

FRACTION	CANE SEGMENT	JUNE 23				JULY 16				AUGUST 9			
		CONTROLS		RINGED		CONTROLS		RINGED		CONTROLS		RINGED	
		Gm.	PERCENT- AGE RESIDUAL DRY WEIGHT	Gm.	PERCENT- AGE CONTROL RESIDUAL DRY WEIGHT	Gm.	PERCENT- AGE RESIDUAL DRY WEIGHT	Gm.	PERCENT- AGE CONTROL RESIDUAL DRY WEIGHT	Gm.	PERCENT- AGE RESIDUAL DRY WEIGHT	Gm.	PERCENT- AGE CONTROL RESIDUAL DRY WEIGHT
Nitrate and nitrite nitrogen	1st.....	0.0080	0.035	0.0110	0.048	0.0085	0.036	0.0108	0.046	0.0016	0.007	0.0043	0.018
	2st.....	0.0135	0.083	0.0003	0.001	0.0181	0.105	0.0181	0.105	0.0080	0.044	0.0042	0.023
	3st.....	0.0139	0.100	0.0094	0.067	0.0149	0.103	.....	.....	0.0048	0.030	0.0063	0.038
	4st.....	0.0221	0.202	0.0105	0.096	0.0213	0.219	0.0118	0.121	0.0096	0.073	0.0038	0.029
	5st.....	0.0117	0.145	0.0289	0.361	0.0213	0.265	0.0136	0.170	0.0040	0.040	.....	.....
	6st.....	0.0160	0.392	0.0092	0.224	.....	.....	.....	.....	0.0032	0.041	0.0072	0.092
	7st.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
	Top.....	0.0276	0.214	0.0122	0.094	0.0085	.....	.....	.....	0.0016	0.025	0.0081	0.128
	7L.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	0.0064	0.042
	6L.....	0.0243	0.144	0.0092	0.054	0.0064	.....	.....	.....	0.0048	0.020	0.0024	0.014
	5L.....	0.0291	0.142	0.0060	0.029	0.0064	0.032	.....	.....	0.0008	0.005	0.0029	0.016
	4L.....	0.0416	0.217	0.0064	0.033	0.0235	0.131	0.0073	0.036	0.0048	0.019	.....	.....
	3L.....	0.0213	0.128	.....	.....	0.0353	0.172	0.0085	0.047	.....	.....	.....	.....

TABLE 5  
DISTRIBUTION OF AMIDE NITROGEN IN NORMAL AND RINGED CANES

FRACTION	CANE SEGMENT	JUNE 23				JULY 16				AUGUST 9			
		CONTROLS		RINGED		CONTROLS		RINGED		CONTROLS		RINGED	
		Gm.	PERCENT-AGE RESIDUAL DRY WEIGHT	Gm.	PERCENT-AGE CONTROL RESIDUAL DRY WEIGHT	Gm.	PERCENT-AGE RESIDUAL DRY WEIGHT	Gm.	PERCENT-AGE CONTROL RESIDUAL DRY WEIGHT	Gm.	PERCENT-AGE RESIDUAL DRY WEIGHT	Gm.	PERCENT-AGE CONTROL RESIDUAL DRY WEIGHT
Amide nitrogen	1st.....	0.0028	0.012	0.0006	0.003	0.0021	0.009	0.0044	0.019	0.0008	0.003	0.0027	0.012
	2st.....	0.0020	0.012	0.0013	0.008	0.0123	0.071	0.0061	0.009	0.0008	0.004	0.0030	0.017
	3st.....	0.0042	0.032	0.0021	0.015	0.0011	0.001	0.0032	0.022	0.0004	0.002	0.0021	0.013
	4st.....	0.0051	0.047	0.0031	0.028	0.0005	0.001	.....	.....	0.0000	0.000	0.0008	0.006
	5st.....	0.0030	0.037	0.0009	0.011	0.0021	0.026	0.0005	0.006	0.0014	0.014	0.0008	0.008
	6st.....	0.0016	0.049	0.0000	0.000	.....	.....	.....	.....	0.0005	0.006	0.0020	0.026
	7st.....	.....	.....	.....	.....	.....	.....	.....	.....	0.0000	0.000	0.0019	0.030
	Top.....	0.0024	0.019	0.0011	0.008	0.0021	.....	0.0017	.....	0.0008	0.005	0.0024	0.016
	7L.....	.....	.....	.....	.....	.....	.....	0.0000	0.0000	0.0005	0.003	0.0020	0.012
	6L.....	0.0012	0.007	0.0004	0.003	0.0005	.....	0.0000	0.0000	0.0004	0.002	0.0015	0.009
	5L.....	0.0020	0.010	0.0005	0.002	.....	.....	0.0011	0.006	0.0004	.....	0.0024	.....
	4L.....	0.0024	0.013	0.0018	0.009	0.0011	0.006	0.0027	0.015	0.0000	0.000	.....	.....
	3L.....	.....	.....	0.0010	0.006	0.0016	0.008	.....	.....	.....	.....	.....	.....

early life of the canes, if for no other reason than that they were no longer so abundantly available to the plant later in the season. The plants were grown in uniform Palouse silt loam from which nitrates are not uniformly supplied; there is an abundant supply of nitrates early in the spring, but this is soon reduced to a low but not deficient quantity.

The effect of ringing on the rise of inorganic solutes in the xylem has been discussed by CLEMENTS and ENGARD (2). They demonstrated that the ring as a break in the continuity of the phloem does not affect the rise of inorganic solutes, but that the ring, as it affects the movement of water, influences the upward movement of salts in the xylem. This effect is produced by prevention of the development of new xylem at that point, which, as growth above continues, becomes a constriction in the cross-sectional area of the conducting channel; also the ringing process causes incapacitation of a portion of the already existing xylem by destruction of cells and clogging of vessels and tracheids upon exposure to air, thus further limiting the capacity of the xylem to conduct water with its dissolved salts. These effects are partly responsible for the erratic figures for nitrate concentration, especially in the first three segments of the ringed canes. Ringing does not prevent the upward movement of nitrates in the xylem, because nitrates are supplied to and utilized by the plant above the rings throughout the period of treatment. The narrow band of xylem which was present when the plants were ringed in May was rather seriously affected by the double ringing. The partial incapacitation of the xylem is shown in the figures for the concentrations of nitrates in the ringed canes of the June collection (ringed May 15). There is an apparent accumulation of nitrates below the rings, a loss between, and a slight loss above. The accumulation appears to be a partial damming of the transpiration stream by the inability of the wounded xylem to conduct the stream. Ringing later in the season (in June and July) is less detrimental to the plant, for, as the data indicate, the figures for nitrate concentration in the first three segments are more nearly normal.

There are other factors which are probably influential in determining the amounts of nitrate in the xylem, as well as in the phloem, in the various parts of the plant. One of these is differential absorp-

tion from the xylem, determined by the rates of reduction and amino acid formation at various places along the stem. Differential growth from one internode to another in part would determine this, and such growth is characteristic of the Cuthbert raspberry. The absorption of nitrates from the xylem would in part depend upon the relative amounts of other substances (especially the carbohydrates and amino acids) present in the various regions of the stem.

**AMMONIUM NITROGEN.**—The concentrations of this fraction throughout the plant are lower than, but parallel to, the alpha amino acids, being highest in the subterminal segment (tables 1, 3), with the tops and leaves very low. In all collections there is an accumulation between the rings, further strengthening the parallelism with alpha amino acids. There is an accumulation of ammonium nitrogen below the rings of the plants of the June collection, and a loss below the rings of the plants collected in August. Deamination of some of the amino acids produced by proteolysis between and below the rings of the June collection accounts for the accumulation of ammonium nitrogen, which apparently does not rapidly escape to the xylem. In the upper regions of the treated plants of the August collection the concentrations of ammonium nitrogen are about normal.

#### NITROGEN GRADIENTS

MASKELL and MASON (9, 10) postulated a relationship between longitudinal movement and concentration gradient. Nitrogen, they found, accumulated above a ring against a strong negative<sup>5</sup> gradient of organic crystalloid nitrogen. They postulated and partly confirmed that this was owing to a small fraction, "residual" nitrogen, which travels downward with a slight positive gradient. The amino acids, which were abundant in the sieve tube layer of the bark of the cotton plant, are thought not to be a translocational form because of the steep negative concentration gradient. The gradient of organic crystalloid nitrogen was positive in the leaves; that is, the concentration was greater in the young than in the old leaves. Total nitrogen was higher in leaves than in bark, but the concentration of organic crystalloid nitrogen was the reverse. Amino acids, plus

<sup>5</sup> Negative is used as opposed to the positive or carbohydrate gradient of higher concentrations in the top, lower concentrations downward.

residual nitrogen, were higher in the leaves than in the bark. In their gradient studies MASKELL and MASON divided the plants into an upper region, in the center of the foliage region, and a basal, non-foliage lower region. Gradients were determined between the two regions and represent the excess of concentration in the upper region over that of the lower region. A negative sign was used to indicate gradients in the opposite direction.

As previously stated, the raspberry plants were separated into 15 cm. segments from bottom to top. The last is a part segment which includes the stem tip and the small unfolding leaves (top). Table 6 presents the total gradient and gradient per centimeter of distance in the normal and ringed stems respectively, and the gradient from the last stem segment (6st or 7st) into the tops for the six nitrogen fractions. The concentrations of total organic nitrogen descend from the highest concentration in the highest stem segment (tops) to lower concentrations in lower stem segments; the gradient is positive downward. The concentrations of the soluble organic nitrogen fractions descend from the highest concentration in the subterminal segment to lower concentrations in the top, the leaves, and lower stem segments; the gradient is positive upward, laterally, and downward respectively.

The leaves and tops are much higher in total nitrogen than any of the above-ground parts. The gradients are steep in the May collection, become less steep in June, and still less by August when metabolism has generally decreased. The gradient per centimeter of stem drops from 0.075 in May to 0.025 in June and 0.004 in August.

The gradients of the total soluble nitrogenous fractions, like those of the total organic nitrogen, exhibit the decrease in gradient per centimeter of stem from May to August. Each fraction contributes to this decrease in the total gradient, nitrates exhibiting the greatest decrease in gradient per centimeter of stem from May to August.

Table 6 presents the effect of ringing on gradients in comparison with gradients in the normal stems. The same general decreases in gradients are shown by these figures as were shown by those of the normal plants, but there are slight modifications. The gradients per centimeter of stem of the soluble organic fractions are greater than

those of the corresponding controls. The gradients in the control and treated plants are less than the beginning control (May) gradients. The gradients of nitrate and ammonium nitrogen are generally less than those of the corresponding controls. There is no effect of

TABLE 6  
CONCENTRATION GRADIENTS IN NORMAL AND RINGED CANES

DATE	NITROGEN FRACTION	NORMAL			RINGED		
		SUBTERMINAL TO BASAL		SUBTERMINAL TO TOP	SUBTERMINAL TO 3ST		SUBTERMINAL TO TOP
		TOTAL GRADIENT	GRADIENT PER CM.		TOTAL GRADIENT	GRADIENT PER CM.	
May 15	Total organic.....	+2.250	+0.075	+0.610*			
	Total soluble organic...	+0.862	+0.029	+1.265†			
	Alpha amino.....	+0.351	+0.012	+0.448			
	Ammonium.....	+0.142	+0.005	+0.383			
	Nitrate.....	+0.410	+0.013	+0.374			
	Diamino.....	+0.010	+0.000	+0.010			
June 23	Total organic.....	+1.908	+0.012	+1.785	+1.240	+0.028	+0.740
	Total soluble organic...	+1.072	+0.014	+0.795	+0.680	+0.015	+0.596
	Alpha amino.....	+0.099	+0.001	+0.025	+0.349	+0.008	+0.280
	Ammonium.....	+0.350	+0.005	+0.294	+0.114	+0.003	+0.126
	Nitrate.....	+0.357	+0.005	+0.178	+0.156	+0.003	+0.130
	Diamino.....	+0.174	+0.002	+0.097	+0.135	+0.003	+0.157
August 9	Total organic.....	+0.355	+0.004	+2.416	+1.036	+0.017	+1.010
	Total soluble organic...	+0.420	+0.005	+0.110	+0.914	+0.015	+0.772
	Alpha amino.....	+0.058	+0.001	+0.012			
	Ammonium.....	+0.055	+0.001		+0.019	+0.000	+0.012
	Nitrate.....	+0.019	+0.000		+0.090	+0.002	+0.086
	Diamino.....	+0.177	+0.002	+0.128	+0.228	+0.004	+0.203

\* Plus (+) = concentration highest at top.

† Plus (+) = concentration lower in top.

ringing on the direction of the gradients between the terminal and subterminal segments, but there is a general reduction in steepness.

The gradients reveal little more than differences in concentrations in the various parts of the plants; changes in gradients reveal only changes in relative concentrations. The decreasing gradient per centimeter of stem indicates a tendency of the fractions to become more nearly equal in all parts of the maturing plant. Ringing modifies the

relative concentrations of nitrogenous substances, probably through modifications in the growth of the upper region of the plant.

The steepness of the gradients of nitrogen fractions in the May plants as compared with those of the June plants would appear to indicate in part a downward movement of nitrogen from the upper regions. The rings, however, would cause an accumulation of a

TABLE 7  
NITROGENOUS SUBSTANCES IN GRAMS IN NORMAL PLANTS OF MAY  
AND NORMAL AND RINGED PLANTS OF JUNE\*

	TOTAL ORGANIC NITROGEN	TOTAL SOLUBLE ORGANIC NITROGEN	ALPHA AMINO NITROGEN	DIAMINO NITROGEN	NITRATE NITROGEN	AMMONIUM NITROGEN
May						
Stem.....	2.149	0.888	0.248	0.059	0.251	0.313
Leaves.....	2.961	0.524	0.104	0.092	0.216	0.083
Total.....	5.110	1.412	0.352	0.151	0.467	0.396
June						
Normal						
Stem.....	1.323	0.423	0.101	0.057	0.112	0.089
Leaves.....	2.448	0.261	0.060	0.045	0.117	0.017
Total.....	3.771	0.684	0.161	0.102	0.229	0.106
Ringed						
Stem.....	1.513	0.435	0.138	0.050	0.081	0.103
Leaves.....	1.842	0.193	0.069	0.029	0.022	0.015
Total.....	3.355	0.628	0.207†	0.079	0.103	0.118

\* Figures are for samples of 12 plants per sample.

† Increase in this fraction produced by accumulation of alpha amino acids between and below the rings.

downward moving organic form of nitrogen in the bark, and the gradient per centimeter of stem above the rings would be reduced greatly. Table 6 shows that the gradient is actually steepened for all fractions except ammonium and nitrate nitrogen.

That there is a considerable loss of nitrogen from the above-ground portion of the plant is shown in table 7, which gives the grams of nitrogenous fractions found in the whole plant (figures obtained by adding the grams per sample figures in tables 1 to 4, each sample representing twelve plants). From May 15 to June 23 there is a great decrease in the amount of all nitrogenous fractions.

The rings had little effect on the amount lost from the above-ground portions of the plant. The grams of nitrogen in the portion of the plant above the rings are compared with the amount of nitrogen in the corresponding portion of the normal plant in table 8. If there were a downward movement of nitrogenous substances in the bark—and substantial movement would be necessary to account for the

TABLE 8

NITROGENOUS SUBSTANCES IN GRAMS IN NORMAL PLANTS OF MAY AND  
NORMAL AND RINGED PLANTS OF JUNE IN PORTION OF PLANT  
ABOVE POSITION OF UPPER RING

	TOTAL ORGANIC NITROGEN	TOTAL SOLUBLE ORGANIC NITROGEN	ALPHA AMINO NITROGEN	DIAMINO NITROGEN	NITRATE NITROGEN	AMMONIUM NITROGEN
May 15						
Stem*.....	0.839	0.258	0.078	0.018	0.090	0.074
Leaves.....	2.961	0.524	0.524	0.092	0.216	0.083
Total.....	3.800	0.782	0.602	0.110	0.306	0.157
June 23						
Normal						
Stem.....	1.034	0.326	0.076	0.047	0.091	0.071
Leaves.....	2.448	0.261	0.060	0.046	0.117	0.017
Total.....	3.482	0.587	0.136	0.093	0.208	0.088
Ringed						
Stem.....	1.150	0.274	0.079	0.038	0.070	0.046
Leaves.....	1.842	0.193	0.069	0.030	0.022	0.015
Total.....	2.992	0.467	0.148	0.068	0.092	0.061

\* These figures represent only nitrogenous fraction in one segment plus stem tip of 10 cm. length or less, whereas figures for stem of June plants represent quantities in four stem segments plus tip.

loss of nitrogen from May 15 to June 23—there would be a definite accumulation above the rings. This did not occur, as table 8 shows, for the amounts of nitrogen above the rings are even slightly lower than those of the corresponding portion (above the position of the rings) of the normal plants harvested at the same time. Table 8 shows that the one segment of the normal May plant, the only segment remaining above the position of the upper ring on corresponding treated plants, contains (with the stem tip) more nitrogen than the four segments and stem tip above the rings of the treated plants of June or of the corresponding portion of the June control plants.

The bulk of the nitrogen in the May plants is in the first two segments (table 1); some of this may be redistributed to the upper segments as the cane increases in length and amount of foliage, and some may be lost either to the roots or to the environment. Instead of accumulation, therefore, there is continuous loss of nitrogen from above the rings, and downward movement in the bark is not involved in the loss.

The loss of nitrogen from May 15 to June 23 is great, of the order of 16–22 per cent, and occurs during the period of most rapid growth.<sup>6</sup> The concentrations in the plants remain at about the same level from May to August, perhaps decreasing a little. The data for all segments of the July and August plants are not complete, so an exact comparison in grams for the whole plant cannot be made, but comparison of the amounts and concentrations in the segments for which there are data bears out this statement. The nitrogen involved in this early spring loss either moves out of the stem to the roots, or is lost to the environment directly from the plant. As pointed out from the data in table 8, there is no accumulation of nitrogenous substances above the rings during the five weeks after ringing, which indicates that there is no downward movement in the bark. It is hardly conceivable that such a great loss of nitrogen from the above-ground portion of the plant took place by ordinary diffusion in the xylem, especially under the conditions of very high transpiration rate which exists during the summer in eastern Washington. A loss of nitrogen of a similar magnitude has been recorded by DONEEN (5) for varieties of wheat grown in this locality, and the loss was assumed to have resulted from translocation to the roots. WILFARTH, RÖMER, and WIMMER (22) reported a loss of nitrogen from several plants. They thought the nitrogen was translocated to the roots, but no analyses were made on the roots. Other investigators have reported losses of nitrogen for which there was no definite accounting. There are several possible ways in which a loss of nitrogen could occur without transport to the roots. One of these is a loss directly to the atmosphere as gaseous nitrogen; the other is washing out by rain.

<sup>6</sup> The plants increased from approximately 50 to 100 cm. in length, and in dry weight from 161 to 202 gm. in the five weeks' interim.

### Discussion

The gradients reported here on the basis of residual dry weight do not indicate the direction of movement or even the existence of a dominant translocational form of nitrogen. The concentration gradients represent little more than gradients of the ratio of living, protoplasm-containing cells to non-living, protoplasm-lacking cells, which is greater in the top of the stem than in any other part. Protoplasm is densest in the meristematic regions, and the highest concentrations of total organic nitrogen are found in the top segment of stem (which includes the stem tip and the young, unfolding leaves) and in the leaves. The protein constituent of the total organic nitrogen is greatest in the top segment and in the youngest leaves. The soluble forms of organic nitrogen are most highly concentrated in the subterminal segment. Amino acids are partly responsible for the soluble nitrogen gradients, their concentration being also very low in stem top and leaves. The protein gradient is the reverse. It appears, then, that the stem tip and the leaves are the greatest protein synthesizing organs of the plant, for amino acids are utilized very rapidly in these regions. The concentration of protoplasm is greatest in these organs.

That the positive gradients do not indicate a downward movement of nitrogen in the bark is revealed by the lack of an accumulation above the rings and by the failure of the gradient to reverse or even to become level. That an upward movement of an organic nitrogenous form in the bark is not important is indicated partly by the lack of a significant accumulation below the rings but more convincingly by the presence above the rings of an amount of nitrogen which is nearly normal. If nitrogen were supplied to the upper regions of the plant chiefly through the bark, upward movement beyond the rings would be prevented and the nitrogen content of the upper region after five weeks would be greatly below normal. Protein synthesis continues above the rings throughout the period of treatment. The nitrate used in this synthesis must have been supplied by the xylem. Apparently nitrate is the important translocational form of nitrogen in the raspberry plant. Nitrates are abundant early in the spring, and rapid utilization throughout the whole

plant results in considerable amounts of organic nitrogenous compounds. The nitrates move freely in the xylem and are available for amino acid synthesis at all points in the plant.

Differential synthesis of protein and utilization of amino acids account for the gradients between stem and leaves and between stem tip and subterminal segment. The same factors are responsible for the descending gradients in the stem, for the amount of amino acid and protein synthesis is proportional to the varying amounts of protoplasm produced throughout the length of the stem. The phloem is probably the most important stem tissue involved in amino acid synthesis. The function of cortex in this process can be ruled out owing to the production of phellem by a pericyclic phellogen early in the life of the plant. Microchemical tests with diphenylamine reveal nitrate in the phloem as well as in the xylem, not only in the raspberry but in other plants as well. Investigations by COOIL (3) in progress in this laboratory reveal nitrate in the phloem exudate of cucurbits. MOOSE (14) has demonstrated in several plants the presence of nitrate and other minerals in the phloem exudate, as well as in the "parenchyma sap" of the leaves. The presence of these inorganic constituents in the phloem he thought was "presumptive evidence that inorganic as well as organic materials are transported in the phloem." It has been demonstrated many times that small quantities of salts move in the phloem, but this movement is of a secondary nature, for, as CLEMENTS (1) has pointed out, the upward movement of soil solutes takes place chiefly in the xylem, downward movement of salts can occur in the phloem, and the bark may obtain salts directly from the xylem as well as from the leaves. The major, rapid movement of salts is in the xylem (1, 2). In addition to these minor salt movements in the phloem, it is well established that metabolically active protoplasm absorbs salts against a strong negative gradient; that is, with an increasing chemical potential. This process is characteristic of absorption of salts by roots; the absorption of nitrates and other inorganic substances from the relatively passive xylem by a metabolically active phloem is a homologous process. Thus lateral absorption from the xylem accounts for much of the inorganic solutes in phloem tissue. In most instances MOOSE (14) observed greater quantities of salts

in the leaf parenchyma extract than in the phloem exudate, which itself included phloem parenchyma juice.

The data presented here for the raspberry plant bear out that part of MASKELL and MASON's assumption which states that the bulk of the mineral nitrogen absorbed by the roots is carried in the transpiration stream. Elaboration of the nitrates to amino acids and protein is not restricted to leaves only, however, but occurs in all living cells of the plant where the proper conditions for nitrate reduction exist. The data for the raspberry do not indicate elaboration in the leaves and exportation through the sieve tubes back to the roots—the second part of MASKELL and MASON's assumption. On the contrary, the data indicate that the amount of synthesis of amino acid and protein is dependent upon the amount and activity of protoplasm, all other conditions for nitrate reduction being favorable, and is not a function of special organs, although in some plants, especially perennials (18), synthesis of amino acids in the roots appears to exceed utilization in protein formation. In such an instance, upward movement of amino acid appears to take place in the phloem.

Although no definite longitudinal polar movement of organic nitrogen is indicated in the bark of the raspberry, the possibility of local movements of organic nitrogenous substances from regions of higher to lower concentrations still exists. Differential utilization of amino acids in various localized regions may result in a movement of this form of nitrogen. Possibly an example of this is a movement of soluble organic nitrogen into the top from the subterminal segment. This movement could be responsible for the gradient of organic nitrogen existing between these regions, but it must be emphasized that the gradient does not indicate that such a movement takes place and may indicate only that differential utilization has occurred. The important mobile form may well be nitrate, which is supplied to both regions. Examples of definite movement of a substance are found, however, in the considerable movements of carbohydrates into ripening fruits, the movement of sugar into the cotton boll (11), etc.

Certainly there is no well founded reason to assume that the

seat of organic nitrogen synthesis is only in the leaves. Nitrogen reduction takes place wherever there is sufficient carbohydrate, reducase, a proper pH, a proper temperature, and essential elements. Many workers, notably ECKERSON (6), THOMAS (18), NIGHTINGALE *et al.* (16, 17), TIEDJENS (19, 20, 21), and LEONARD (8), have shown that synthesis may take place to great extent in organs other than the leaves.

### Summary

1. Data are presented concerning the distribution and translocation of total organic, total soluble organic, alpha amino, diamino, amide, ammonium, and nitrate nitrogen in the vegetative canes of the Cuthbert raspberry. Double rings were used to check the movement of nitrogenous substances, one ring 15 cm. and the other 30 cm. above the ground. The plants were cut for analysis into 15 cm. lengths. Leaves were analyzed separately from stem segments.

2. There was a steady increase in the concentration of total organic nitrogen from the basal segment to the top. Total soluble organic nitrogen and alpha amino acids attained a maximum concentration in the subterminal segment. Total organic nitrogen was higher in the leaves than in the stem, but the total soluble nitrogen and amino acids were about 50 per cent lower in the leaves than in the stems. The concentration of these substances was about 50 per cent lower in the terminal than in the subterminal stem segment. Protein was therefore most abundant in the stem top and leaves.

3. Nitrogenous compounds did not accumulate in the upper regions of the ringed plants. There was not a significant accumulation below the rings. Increase in the concentration of soluble nitrogen in the necrotic segment between the rings was owing to production of amino acids by proteolysis.

4. All nitrogen gradients were positive, and are thought to be little more than manifestations of the ratio of the amount of living, protoplasm-containing cells to non-living, protoplasm-lacking cells in the various parts of the plant. Thus the concentration of total organic nitrogen is greatest in the top segment and leaves, where there is least differentiation and thickening of cells and much meri-

stematic tissue. The gradient of soluble nitrogen from subterminal to top segment (positive upward), and from stem to leaves (positive laterally), seems to be a manifestation of the differential utilization of the soluble forms, with the greater amount of protein synthesis taking place in the top and leaves, rather than an indication of movement of nitrogen. The gradients in the stem (positive downward) are the result of synthesis or accumulation of nitrogenous substances in the living cells.

5. There appears to be considerable loss of nitrogen from the above-ground portion of the plant from May 15 to June 23. Since there is no accumulation above the rings, the 16-22 per cent loss cannot be explained by movement to the roots in the bark. It is hardly conceivable that it was translocated to the roots in the xylem, in which the transpiration stream is under a great negative tension owing to a high rate of transpiration. It is suggested that the loss is in the form of a gas, or occurs through leaching by rain.

6. It is concluded that there is no definite longitudinal translocation of organic nitrogen either upward or downward in the bark. Nitrate is the important translocational form of nitrogen in the Cuthbert raspberry. It rises freely in the xylem, and is available for reduction and elaboration to amino acids and protein not only in the leaves but in all living cells of the plant where conditions favorable to reduction exist. The amount of nitrate reduction and elaboration is proportional to the amount of protoplasm in the various parts of the plant; thus the greatest amount of protein is found in the stem top and leaves. Reduction and synthesis take place also in the stem and in proportion to the concentration of protoplasm per unit of weight; hence the existence of a positive concentration gradient.

7. In the stem the phloem is a highly metabolic tissue, and absorbs nitrates and other inorganic salts from the relatively passive xylem. The presence of inorganic solutes in the phloem is the result of an absorption process homologous with the absorption of salts by roots, and is not a manifestation of primary solute translocation.

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# ANATOMY OF THE AERIAL AXES OF *EQUISETUM KANSANUM*

ISABEL M. P. BROWNE

(WITH ONE FIGURE)

## Introduction

In 1912 Professor J. H. SCHAFFNER described as *E. kansanum* a new species of *Equisetum* that had until then been confused with *E. laevigatum* A. Br., from which it differed chiefly in that its aerial stems were annual and its cones devoid of a specialized, rigid apical point. The specific diagnosis ran: "Aerial stems usually 1-2½ ft. high, annual, very smooth, 15-30 grooved,<sup>1</sup> usually without simple branches, unless broken off; color mostly light green; surface of the ridges and grooves with cross and diagonal bands; sheaths long, dilated above and usually constricted at the base, green with a narrow black band at the top; teeth deciduous; cones ovate or oblong-ovate, without a point, the apex obtuse or merely acute" (4, p. 21).

SCHAFFNER most kindly sent me from Ohio material of his new species, duly fixed and preserved for anatomical investigation. He was also good enough to discuss with me by correspondence the nature of the new species. For these privileges I wish to thank him sincerely.

The following short account of the anatomy of the aerial stems of *Equisetum kansanum* Schaffner is based on microtome sections of nine axes.<sup>2</sup> Serial transverse sections were made of two large, of one medium sized, and of two small stems. Of one of the large stems the series extended from the upper end of a node downward through that node, through a complete internode, through a second node, and through part of the internode below the latter. In the other cases the portions included a complete node with portions of the inter-

<sup>1</sup> In the largest of my specimens (fig. 1), thirty-seven grooves and bundles were observed.

<sup>2</sup> Some of the smaller axes sent me may have been branches, but this is unlikely, as branching of aerial stems is rare. Moreover, all but one of these small axes bore cones, and there seems to be no record of fertile branches.

nodes above and below. The three small branches were fertile and in each case the node sectioned was the uppermost vegetative one. Serial longitudinal sections were made through four axes. In all of them the part sectioned included a complete node with portions of the internodes above and below. One of the stems was very large, one was of medium size, and two were very small. One of the latter was fertile and in it the node sectioned was the uppermost vegetative one.

### Observations

#### LARGE STEMS

Stem *A*, the largest of those cut transversely, had thirty-seven bundles and ribs, and at its widest was 6 mm. in diameter. At the level of the diaphragm it narrowed, the leaf sheath being constricted at the base to a diameter of less than 5 mm. The other large axis cut transversely, stem *B*, had thirty-two ribs and bundles below the lower of the two nodes. When the ring of nodal xylem broke up, only twenty-nine bundles were reconstituted, each lying opposite a rib. This was also the number of leaves and leaf traces at the next node; but, rather surprisingly, on the breaking up of this (upper) nodal ring of xylem thirty-two bundles were once more reconstituted, each lying opposite a rib. A few of these were unusually small and it is possible that some of them died out a little higher up, although the sections did not extend far enough to show whether this was so. At its widest, stem *B* attained a diameter of about 4.5 mm., while at the level of the diaphragm it was but 4 mm. wide. In the large stem sectioned longitudinally, stem *C*, the axis at its widest was about 4.25 mm., while at the level of the diaphragm it was only about 2.75 mm. wide. This stem was very young, and above the diaphragm the tissues were immature for a distance of about 6 mm. At this level the axis was in a very youthful phase, most of its cells being small and in process of rapid division.

As in *Equisetum laevigatum*, the furrowing of the stem is not very conspicuous, the grooves being wide and shallow. The ribs are rounded and externally convex, without indication of the biangular form with slight median concavity found in *E. laevigatum* (2, pl. xxxii, fig. 3). *E. kansanum* is one of the *Equiseta* cryptopora, species

with stomata sunk beneath the surface of the epidermis. The stomata are disposed in two uniseriate rows in each groove, each row lying on one side of the latter, about halfway between its middle and the middle of the neighboring rib. The stomata of a row are separated from one another by a single short cell. Each stoma abuts on a schizogenously formed air space.

In the mature regions of the internode, two to three rows of long, narrow, pointed fibers lie immediately under the epidermis. Under the ribs the fibers are more numerous, and so disposed that in transverse section they form a triangular tooth, projecting inward toward the bundle. In a radial direction this tooth may contain from six to twelve fibers. Shorter teeth, composed of similar fibers, sometimes project inward from the middle of grooves, although in this position but few additional fibers are developed and the teeth rarely attain more than half the radial length of those under the ribs, while they are often completely absent. The extent of development of the fibrous teeth under the ribs and grooves varies very much, not only in different stems and different parts of the same stem, but even in adjacent ribs and grooves of the same section.

Since in *Equisetum*, differentiation of the tissues proceeds in each articulation from above downward, the cells just below the node are often in a more mature condition than are those lower down in the internode; and at this level the tip of a projecting tooth of fibers under a rib may be connected with the bundle on the same radius by a single (rarely locally double) chainlike row of somewhat radially elongated cells with slightly thickened walls. It seems to be rare to find this condition in neighboring bundles, and in my specimens at least, such a row of cells had a very slight vertical extension—not more than  $70\ \mu$ . A similar development occurs, although rarely, lower down in the internode. One of MILDE's figures of *E. trachyodon* A. Br. shows a very similar conformation of the cells (2, pl. xxxiii, fig. 14).

The assimilating tissue (MILDE's *grüne Parenchymschicht*) lies immediately under the subepidermal layers of fibers. It consists of two to four (usually three) rows of cells with thin undulating walls. These cells, which are differentiated at an early stage, have dense contents and form a loose spongy tissue. Those of the innermost row

are more or less isodiametrical and close to one another. Those of the outer two rows are radially elongated and between them are developed schizogenous air spaces wider than the cells. In a longitudinal section of the stem these layers have a very characteristic, ladder-like appearance; although when, as is frequently the case, the middle cell of the three joins on to two cells (one belonging to its own and one to a neighboring row), the bars of the ladder seem to fork. In longitudinal section these bars always seem to slope obliquely upward, presumably an effect of growth. As seen in a transverse section of the axis, the external outline of the assimilating tissue is determined by the absence or presence and degree of development of the fibrous toothlike projections. Opposite the ribs of the stem it extends in most cases inward to within a layer or two of the outer endodermis of the bundle; opposite the grooves it is generally bounded internally by the outer margins of the vallecular canals. Even before the latter are formed, their limits are distinctly visible. The cells that are later destroyed by the formation of the vallecular canals are much larger than those of the assimilating tissue, and begin to lose their contents very early. In a transverse section of the stem they form oval tracts of thin-walled, collapsible looking tissue, of which the middle cell is the largest and also the first to be destroyed. In a typically internodal section the vallecular canals are from two to four times as wide transversely as radially; but as the node is approached and the bundles increase in size, the canals become narrower.

In the internode, outer and inner ground tissues are completely separated by an endodermis or rigid sheath of small cells, the radial walls of which do not show the typical endodermal markings. This external endodermis runs round the periphery of each bundle and follows the contour of the latter for some distance, usually until it reaches about the depth of the middle of the carinal canal. It then bends more or less at right angles and runs to the next bundle, and bending again outward, follows the outer part of the contour of this bundle. The outer endodermis thus surrounds the peripheral part of each bundle in succession. There is also an inner endodermis running along the inner edges of the bundles; this too is continued between

the bundles, either running conformably to the circle in which the latter are arranged or curving a little outward between them. The cells abutting on the sides of the bundles and lying between the outer and inner endodermes also develop as part of the sheath. In this region and at the periphery of the bundles the outer endodermis often becomes two cells thick, the cells of the outer row being rather the larger. On looking at a transverse section of the stem, all three forms of endodermis—outer, inner, and special—seem to be developed.

As early as 1867, PFITZER gave a clear description of the inner and outer endodermes that he had found in the stems of *E. hyemale* L., *E. trachyodon* A.Br., *E. ramosissimum* Desf., and *E. variegatum* Schleicher (3, pp. 310-312). As can be seen by comparing his figure 8 of pl. xviii showing a transverse section of two bundles from the internode of *E. variegatum* with my figure of two bundles of the internode of *E. kansanum*, it would be necessary only for about two cells on each side of the bundle, near its inner edge, to be differentiated as cells of the sheath to convert the type of sheath figured by MILDE into the kind found in *E. kansanum*. In the latter species the endodermal sheath system seems to be much more developed than in the species with inner and outer endodermes, described by PFITZER. He stated that in the region between the bundles, inner and outer sheaths were usually separated by one or more (rarely by two) layers of cells, but that in a few cases they were in contact (3, p. 311). In *E. kansanum* inner and outer endodermes are in contact throughout their course; and a third—or even locally a fourth series—of cells may be added to the sheath, either internally or externally, or on one or on both sides and for part or for the whole of the distance between two bundles.

In a longitudinal section it can be seen that when mature, the internodal cells of the sheath are vertically elongated, although very narrow radially. Transverse sections show that between the bundles the cells of the sheath are somewhat elongated tangentially, although not nearly so much so as vertically. Even vertically they are not so long as the tracheids and their ends are blunt, the transverse

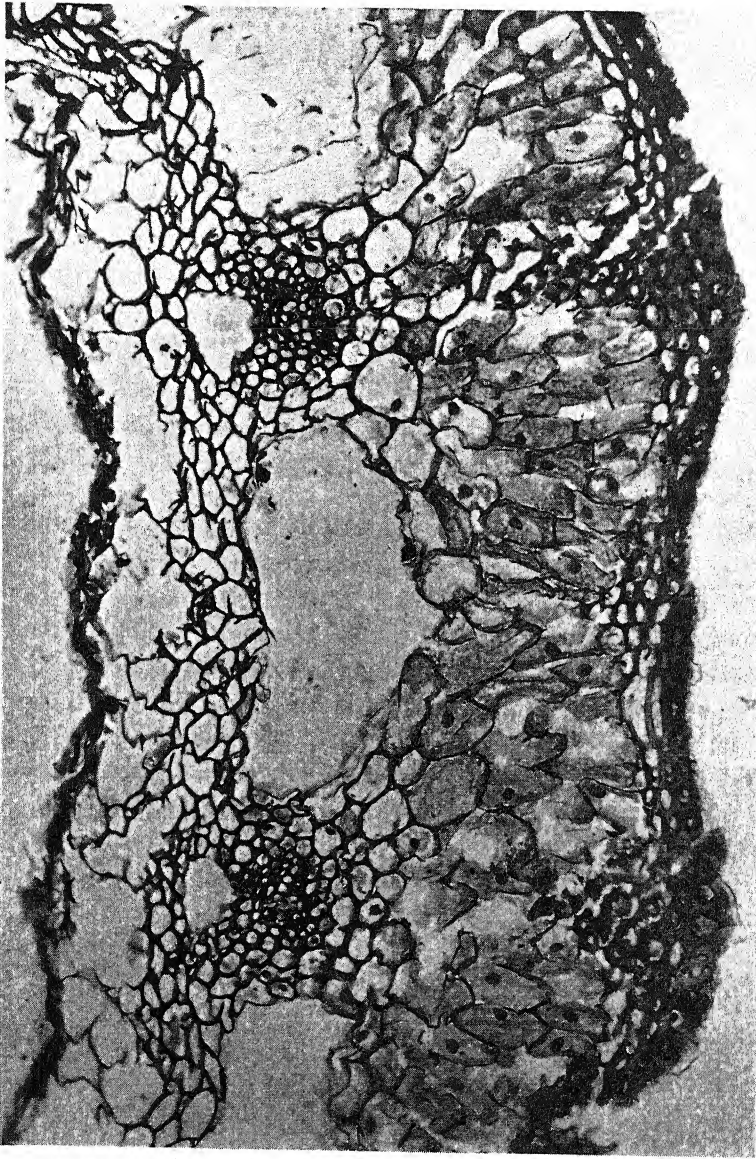


FIG. 1.—Transverse section from large stem of *E. kansanum*. Note inwardly projecting teeth of fibers of unequal length under ribs and grooves, and row of cells, somewhat elongated radially and with slightly thickened walls, connecting tip of one tooth with bundle on same radius. Section traverses two stomatal apparatus (which lie in shallow furrows on either side of middle of groove) at different levels. On right the section passes through part of a schizogenous air space below a stoma. Note well developed multiseriate endodermis and, abutting on central cavity, disorganized remains of cells destroyed by formation of that cavity. These remains consist chiefly of walls of collapsed cells and probably correspond to tissue erroneously interpreted and figured by MILDE as a narrow band of delicate or gauzelike cells delimiting the central cavity.

walls being commonly somewhat oblique. After staining, these walls show numerous small, irregularly placed light spots, probably pores.<sup>3</sup>

Except in the neighborhood of the nodes, the bundles are oval in outline, with the longer axis directed radially, and far apart, the distance between them being from two to four times as great as their own width, or sometimes even more. In mature regions of the internode the carinal canal is often relatively large and may occupy half, or more, of the space within the sheath of the bundle. There is great variety in the shape and size of the carinal canals. In two adjacent bundles of the same section one carinal canal may be half the size of its neighbor, and round or oval; if oval, the longer axis may be radial or transverse. The metaxylem elements are usually arranged in two nearly radial rows, each row abutting on the lateral part of the sheath surrounding the bundle and curving slightly inward, conformably to the shape of the sheath. Typically the rows of metaxylem are but one tracheid in width; frequently there are two tracheids, side by side, at the inner end of a row; and not infrequently one and occasionally both rows are replaced by small groups of tracheids. But whether the tracheids are in a row or a group, the narrower end of the metaxylem is toward the periphery of the bundle. Here there is a single narrow tracheid. Each row or group consists of from two to seven elements, four to six being the usual number. Sections through the regions where the metaxylem is not yet completely developed show that the direction of differentiation is centrifugal, as it is in all the species (*E. arvense*, *E. palustre*, *E. limosum*, *E. maximum*, *E. hyemale*, *E. sylvaticum*, *E. debile*, and *E. variegatum*) in which this point has been elucidated (1, pp. 458-459) except *E. giganteum*. It is remarkable that this centrifugal development seems to be less subject to irregularities in *E. kansanum* than in the other species studied, and this although the diameter of the metaxylem tracheids decreases steadily and markedly in size from within outward.<sup>4</sup> It would seem that the more peripherally

<sup>3</sup> Similar but less well marked pores were observed on the transverse walls of some of the larger cortical cells abutting on the endodermal sheath. PEITZER (3, p. 333) appears to have observed somewhat similar small pores on the vertical walls of the cells of the sheath and of the parenchymatous tissues at the node.

<sup>4</sup> In a transverse section of the stem it sometimes seemed as though a more deeply seated tracheid were smaller than a more peripherally situated one, but this was due to the plane of the section passing through either of the narrow ends of a tracheid.

situated cells of the bundle divide more frequently and more rapidly than those toward its interior, and are thus considerably smaller than the latter at the time of their lignification.

The lateral groups of metaxylem are differentiated much later than the protoxylem, and the latter has usually been replaced by a carinal canal before the first metaxylem tracheid is differentiated. Occasionally one group of metaxylem develops before the other; and apparently in some cases one group failed to differentiate at all over a short vertical distance (*cf.* 2, pl. xxxiii, fig. 15, *E. trachyodon*). In mature parts of the internode, both the phloem and the parenchyma lying between the lateral bands of metaxylem may become lignified. In mature regions of the internode the central cavity occupies four-fifths or an even higher proportion of the axis. It forms very early, while the cells which will be replaced by vallicular canals are still indistinguishable from their neighbors. Destruction of the centrally seated parenchyma of the stele and of the protoxylem begins at the same level, a little above the diaphragm, while the cells of the ground tissue are still in a very youthful condition. Near the center of the axis a few torn remains of ground tissue persist above the diaphragm. The cavity widens rapidly; and while the epidermal and cortical tissue and that of the vascular bundles (with the exception of the protophloem and protoxylem) are highly immature and the cells composing them are still undergoing division, the whole of the protoxylem and all the ground tissue internal to the bundles—except four to seven rows immediately abutting on the inner endodermis—have perished. As a rule, even in mature stems two to three rows of large thin-walled cells persist inside the inner endodermis, although they too may sometimes disappear for a short distance. The collapsed remains of some of the last cells to be destroyed cling to the edge of those that persist, inside which they form a continuous, narrow band. As the walls are the portions of the cell that resist destruction longest and as they are deposited in layers but slightly separated from one another, their appearance is not unlike that of layers of narrow delicate cells. MILDE has figured and described such layers of approximated, delicate, thin-walled cells bordering on the central cavity in *E. myriochaetum*, *E. hyemale*, *E. laevigatum*,

and *E. trachyodon*.<sup>5</sup> MILDE (2, p. 516) calls the cells of these layers delicate, or very or extremely delicate, even gauzelike (*florartig*). It seems unlikely that destruction of the ground tissue would be arrested at a spot where the nature of the cells is such that they would offer very little resistance. Therefore, and because of the similarity of his figures of these layers to the layers occupying the same position in *E. kansanum*, I think it probable that MILDE, working with herbarium material, mistook such torn remains of cells for intact layers of narrow, delicate cells. His figure of *E. trachyodon* seems to be the one which in regard to the tissues between the endodermis and the central cavity comes nearest to *E. kansanum*. Here, as is common in *E. kansanum*, a few layers of large thin-walled cells persist internally to the endodermis.

#### NODAL STRUCTURE

At the level of the node of stem *A* one, at that of the lower of the two nodes of stem *B* three, and at that of the node of stem *C* two, branches were initiated. None of these branches broke through the leaf sheath and their presence was revealed only by study of the sections. No material difference was observed in the manner of attachment of the vascular supply of these branches to the axial stele from what has been recorded for the genus; and in the following short account of the nodal structure of *E. kansanum* the complications introduced by the presence of these dormant branches, as well as those due to a change in number of the bundles of the successive nodes and internodes of stem *B*, will be left out of account. The irregularities resulting from these causes are confined to a relatively narrow sector of the stele.

As the node is approached from below, the vascular bundles and the tracts of persistent parenchyma connecting them with the fibers under the ribs increase in width. The vallecular canals consequently become narrower, and for a distance below the node their longer axis is radial. Higher up, but still below the level of the diaphragm, the outline of the vallecular canals becomes for a short time circular.

<sup>5</sup> Cf. my figure 1 and 2, pp. 498, 516, 549, 560; and pl. xxvii, fig. 6; pl. xxix, fig. 19; pl. xxx, fig. 23; pl. xxxii, fig. 5; and pl. xxxiii, fig. 14. In the case of *E. trachyodon*, the word used by MILDE for approximated is *genährte*, clearly a misprint for *genäherte*.

This is due to the fact that vertically above the space occupied by the inner parts of the vallecular canals (which through most of the internode reach inward to about half the radial depth of the bundles) nodal tracheids are developed by the enlarging vascular bundles.

Within the bundle the processes at the node resemble in essentials those described for other species of *Equisetum*. That is to say, the tracheids in the lateral groups of metaxylem increase in number and in size; a transverse bar of xylem develops, joining up the two lateral groups, and this gradually thickens until the metaxylem forms an oval mass. The tracheids are of the usual nodal type found in *Equisetum*—short, wide, and reticulately thickened. Meanwhile the carinal canals have disappeared, nodal tracheids occupying their place, except where the protoxylem destined to the trace persists—in a median position at the inner edge of the bundle. Before departure of the protoxylem, a narrow parenchymatous gully forms, running through the middle of the bundle. This gully develops from within outward and passes through the bundle obliquely upward. The protoxylem passes slowly and steeply upward and outward through it, the peripheral part of the nodal xylem being still unbroken when the protoxylem is passing through the innermost part of the gully. Typically all the protoxylem passes out into the trace, and as seen in transverse section of the latter there are about six to eight elements of protoxylem. Once or twice a tracheid or two seemed to be left behind in the axis; then such elements died out almost at once. The protoxylem of successive internodes is therefore completely discontinuous. Some of the nodal tracheids from the sides of the parenchymatous gully, near its outer end, often pass out in the trace; but the xylem of the latter is chiefly composed of protoxylem. Each trace is surrounded by a separate sheath, or endodermis, and this is derived from the external two-layered endodermis of the axis. As the traces pursue a steep outward course through the cortex, the abaxial halves of their sheaths are continuous with the outer layer and the adaxial halves with the inner layer of the two-rowed external endodermis of the stem.

It seems characteristic of the large axes of this species that full development of the nodal xylem is not attained until after the protoxylem of the trace has become completely free from the axial

xylem. During the slow progress of the protoxylem through the parenchymatous gully, the bundles are in contact laterally by their endodermes. The endodermal cells on the sides of the bundles are subsequently replaced by nodal tracheids and the xylem of neighboring bundles becomes confluent. As the nodal xylem is of slight vertical extent and as the bundles do not all develop their nodal xylem at exactly the same level, no single transverse section of the axis shows a complete ring of nodal wood. When the trace is set free from the axial stele, the inner and outer layers of the external endodermis unite round it, so that it is as it were nipped off. Opposite the points of departure of the traces, the nodal xylem becomes thinner radially, and at these points it breaks somewhat higher up, the newly constituted bundles alternating with those of the internode below. When the nodal ring of xylem breaks up, inner and outer endodermes become invaginated and meet round the bundles, so that these when first constituted form transversely elongated oval masses, separated from one another by a single row of endodermal cells. Soon this layer duplicates, and the resulting layers themselves duplicate, so that the xylem of adjacent bundles is for a time separated by four endodermal cells. Parenchymatous cells then appear between the double endodermes; and the bundles, steadily narrowing, come to lie farther and farther apart.

There seems to be some variety as to the level at which protoxylem appears in the internode, but in my specimens it did not do so until after the bundles with their separate endodermes had been reconstituted; it usually reappeared from  $200\ \mu$  to approximately  $220\ \mu$  above its disappearance into a trace. Soon after the appearance of the first nodal tracheids, narrowing of the outline of the circle formed by the xylem of the axis begins. This narrowing continues in passing upward, until at or slightly below the level of the diaphragm the diameter of the circle formed by the xylem is at least 1 mm. less than at a definitely internodal level. This is well seen when a longitudinal section passes more or less radially through a bundle; the latter then seems to be terminated just below the diaphragm by an incurved, club-shaped mass of short, wide, reticulate tracheids, these forming part of the ring of nodal tracheids.

The sclerized diaphragm is situated at the level at which, after

the traces have become free from the axial stele, the nodal xylem is dying out in the reconstituted bundles and being replaced by carinal canals, containing as a rule remains of protoxylem. It consists of two layers of cells (although in parts only one of the two superposed cells is definitely sclerized), except at the periphery, where there seems usually to be but one sclerized layer. The cells of the diaphragm are vertically very short but tangentially elongated, so that in a longitudinal section of the stem they seem brick-shaped. As early as 1867 PFITZER (3) noted that at the node the various types of cell became shorter and had thicker walls. A few remains of parenchymatous cells persist just below the diaphragm, and toward the periphery of the central cavity they are rather more numerous and less torn. These cells are also somewhat shortened, but not so much so as the cells of the diaphragm, from which they are sharply differentiated.

In the largest axis studied the diaphragm became gradually more and more depressed, although but slightly so, toward the center of the axis. Consequently no single transverse section of the latter showed the central cavity completely replaced by the diaphragm; and, in a series of transverse sections passing from below upward through the node, sclerization of the cells seemed gradually to spread outward. As the result a sclerized sheath is formed round the reconstituted bundles of the stele. The sclerized band then ceases to be formed inside the bundles; and the sclerization still spreading outward, the band of sclerized cells comes to lie between the outer edges of the bundles and the traces. These latter are themselves moving slowly and steeply outward.

Soon after this phase has been attained, the innermost layer of the sclerized band, with all the tissue external to it, separates from the axis to form the free part of the leaf sheath. This innermost layer becomes the adaxial (morphologically upper) epidermis of the leaf sheath. At the level at which this separation took place, the outer part of the axial tissues was, in all my specimens of large axes, in a highly immature condition. Its cells had thin walls and were almost completely filled by large, deeply staining nuclei. This is, in fact, the base of the internode, which in *Equisetum* is the region where cell differentiation is longest deferred. Since all the tissues vertically

above the cortex and vallecular canals of the internode below have become separated off as part of the leaf sheath, the axis suddenly becomes much narrower above the diaphragm. As the leaf sheath widens, the stem again widens; epidermis, green parenchyma, and other tissues are again gradually differentiated; and the stem once more attains its ordinary internodal size and appearance.

#### SMALLER AERIAL AXES

In smaller stems definite inwardly projecting teeth of hypodermal fibers do not develop under the ribs and furrows. In the internodes the central cavity is still very wide, occupying usually from two-thirds to four-fifths of the whole axis; but relatively as well as actually it is smaller than in the largest axes. The vertical extent of the nodal xylem is markedly less, and the protoxylem of the trace seems to pass out more rapidly, pursuing a much less oblique upward course through the bundles. The amount of persistent tissue just below the diaphragm is relatively greater in these small stems. The diaphragm, too, seems often to be somewhat raised at the center of the axis, so that it has the shape of a low dome. A series of longitudinal sections passing through a cone, the peduncle of the latter, and the uppermost vegetative node revealed the presence in this specimen of a small rounded protuberance of parenchyma, seated on the central highest part of the low dome-shaped diaphragm. In the smaller stems also, the axial protoxylem of successive internodes is completely discontinuous. In the cone-bearing specimen just mentioned, the tissues, even immediately above the diaphragm, were fully mature; but there was a vertical distance of about 0.5 mm. from which protoxylem was absent, the only axial xylem present being nodal tracheids.

In the smaller stems, at least in my specimens, the branches attained a relatively greater development. Two cases were observed in which the tip of a branch just broke through the leaf sheath, although even in these cases they developed no further; for in none of my specimens were there any of those simple branches recorded by SCHAFFNER in his diagnosis (4, p. 21). In other cases the leaf sheath bulged markedly outward and the exterior of the axis inside it was deeply indented opposite the dormant branches, so that the

latter could fit in between stem and leaf. In several cases the axis became for a time oval or irregularly heart-shaped, as seen in transverse section, owing to the pressure of the branches.

It is curious that at the level of the four nodes of the largest stems—those in which there were thirty or more bundles—only five branches were initiated, two of the nodes showing no dormant branches. At the four nodes with from fourteen to twenty-three bundles, nine branches were initiated, each node showing at least one dormant branch; some two, and some three. At the level of each of the nodes of the two smallest stems, with eight and ten bundles respectively, a single branch was initiated.

### Discussion

This short account of the anatomy of *Equisetum kansanum* supports the view that this plant is specifically distinct from *E. laevigatum*. Anatomically *E. kansanum* appears to be nearer to *E. hyemale* and to *E. trachyodon* than to *E. laevigatum*. The similarity to *E. hyemale* is more particularly to the variety *doellii* (2, pl. xxix, fig. 19), since in the latter there is a rigid sheath of small cells separating outer and inner ground tissue. But of all the species the anatomy of which has been described, it is *E. trachyodon* that anatomically comes nearest to the larger axes of *E. kansanum*. The form of the sheath separating inner and outer ground tissue, the narrow oval outline of the bundles with their long axes directed radially, even the triangular form of the projecting teeth of hypodermal fibers under the ribs of the stem (as seen in transverse section of the axis) and the presence under some bundles of the larger stems of a radial row of cells with slightly thickened walls leading from the inwardly directed apex of the tooth to the periphery of the bundle,—all these can be seen in MILDE's figures of *E. trachyodon* (2, pl. xxxii, figs. 14, 18). Again, MILDE figures a bundle (pl. xxxiii, fig. 15) in which only one of the lateral bands of metaxylem has developed, an irregularity noted as occurring locally in *E. kansanum*. However, in *E. kansanum* the central cavity occupies an even larger proportion of the whole axis than in *E. hyemale* and in *E. trachyodon*; and the ribs of the stem of *E. kansanum* are slightly convex, and not, as in the other two species, concave with two lateral angles.

The smaller stems of *E. kansanum*, those with from eight to twenty-three bundles, do not show the same similarity to the stems of *E. trachyodon*, since there are in them no inwardly projecting teeth of hypodermal fibers or rows of cells with thickened walls running from these to the bundles. This is curious, because in size *E. trachyodon*—with eight to fourteen bundles and ribs—comes nearer to the smaller than to the larger axes of *E. kansanum*.

In 1867 MILDE, while maintaining that *E. trachyodon* was a good species in the same sense as were *E. ramosissimum*, *E. variegatum*, and *E. scirpoides*, admitted that it certainly passed over into *E. hyemale*; and he mentioned the variety *doellii* of *E. hyemale* as being such a transitional form. In describing var. *doellii* itself, however, MILDE, while repeating the statement that this variety represented a transition (*Übergang*) to *E. trachyodon*, added: "it can, nevertheless, be distinguished from *E. trachyodon*, in company with which it alone occurs, by its more vigorous growth, narrower ribs, . . . by the siliceous ornamentation of the epidermis of the stem and by the form of the leaf sheath, after the shedding of its teeth" (2, pp. 519, 563). In 1930 SCHAFFNER stated that he was unable to come to a conclusion as to the specific validity or hybrid nature of *E. trachyodon*, but that there were forms going under varietal names of *E. hyemale* which seemed to have their real affinity with *E. trachyodon*; and that when these were grouped together they made a respectable species of wide distribution in the northern part of the north temperate zone (7, p. 101). In 1932 SCHAFFNER, whose knowledge of these species of *Equisetum* in their natural habitat is probably unrivaled, took the step of placing *E. trachyodon*, including var. *doellii*, with *E. variegatum*, *E. nelsoni*, and *E. scirpoides*, in his *Equiseta pusilla* (8). This group, he believes, separated from the lower *Equiseta hiberna* "quite early in the evolutionary history, there being no direct living transition species" (6, p. 14). SCHAFFNER's group of *Equiseta hiberna* includes *E. hyemale*, *E. ramosissimum*, *E. debile*, *E. laevigatum*, *E. praealtum* (= *E. robustum*), *E. moorei*, and *E. myriochaetum*.<sup>6</sup> In 1912, when SCHAFFNER first made *E.*

<sup>6</sup> In 1925 SCHAFFNER placed *E. myriochaetum* among his *Equiseta primitiva*; later he transferred it to the *Equiseta hiberna*, considering it as the lowest species of that group (5, 8).

*kansanum* into a separate species, he noted that in size and manner of growth it was closely allied to *E. hyemale* and to the larger variety *E. robustum* (4, p. 21); and in 1925 he placed *E. kansanum* and *E. funstoni* at the two ends of a forking line originating farther back than the point of division of the two groups which, in his publications since 1930, he has distinguished as *Equiseta hiberna* and *Equiseta pusilla* (5, pl. iii; 8, p. 14). In 1930 he held that *E. kansanum* was probably derived from *E. laevigatum* (6, p. 16). In view of the fact that, until the date of its recognition as a separate species, the former was usually regarded as an annual form of the latter, this is not surprising. While anatomical characters cannot by themselves claim to be decisive in regard to systematic position, and still less in regard to the bewildering problems of phylogeny, the anatomy of *E. kansanum* does suggest the possibility that the nearest affinity of this species may be either with *E. trachyodon* or with *E. hyemale*, through a somewhat larger form than the typical *E. trachyodon*, that is, through the form known to MILDE as var. *doellii* of *E. hyemale*, a variety which SCHAFFNER places in *E. trachyodon*.

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# CHROMOSOME NUMBERS IN ROOT NODULES AND ROOT TIPS OF CERTAIN LEGUMINOSAE<sup>1</sup>

LOUISE WIPF

(WITH THIRTY-EIGHT FIGURES)

## Introduction

An earlier paper (56) reported the chromosome numbers in infected cells of root nodules of red clover, common vetch, and garden pea to be twice the typical somatic number for these species. Further examinations have been made to determine: (1) whether a similar relationship holds in other species; (2) whether it holds consistently in genera presenting polyploid series; (3) the chromosome condition in plants in which nitrogen fixation has been substantially inhibited either in the presence of ineffective strains of *Rhizobium*, the presence of combined nitrogen in the nutrient, or by treatment with hydrogen or with carbon monoxide.

Chromosome numbers associated with bacterial infection in root nodules have heretofore received little attention. FRED (19) noted that the mitotic figures in the cells of the nodules of some fifty leguminous plants, and particularly of the white lupine (20), "are larger, very irregular, and not well marked, with uneven number of chromosomes" as compared with the cells of the root tips. This irregularity of chromosome numbers suggested that the nodular tissues may be analogous to diseased tissues of animals. DANGEARD (14) described enlarged nuclei in the infected cells of several of the Leguminosae. His chromosome determinations were only approximate, and no mention was made of a regularly increased number. He showed, however, a polar view of an equatorial plate with 26 chromosomes from a nodule of *Trifolium pratense*. This, as now

<sup>1</sup> Herman Frasch Foundation in Agricultural Chemistry, Frasch Paper no. 184. Joint contribution from the departments of agricultural bacteriology and from the department of genetics, Agricultural Experiment Station, no. 240, and the department of botany, University of Wisconsin. Published with the approval of the Director of the Station.

known, is approximately the  $4n$  number, 28. MILOVIDOV (39) found 42 chromosomes in cells of the cortex of the nodule of *Lupinus mutabilis*. LECHTOVA-TRNKA (35) reported approximately 16 as the chromosome number in nodular tissues of both *Sophora moorcroftiana* and *Robinia viscosa*.

### Material and methods

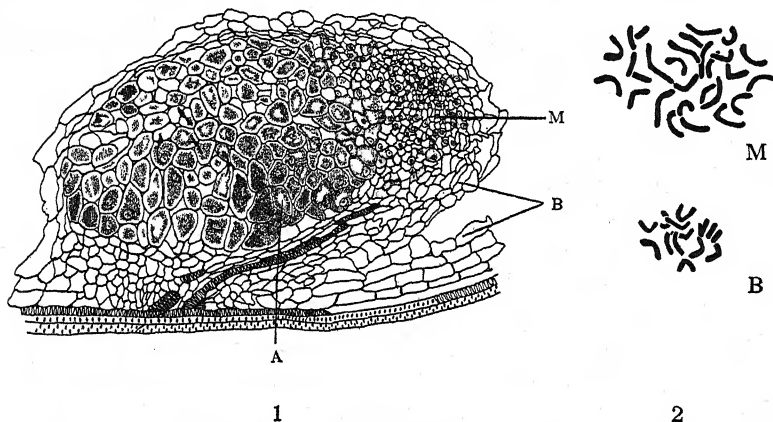
The nodules and root tips used in these examinations were obtained from plants growing in both controlled and natural environments. Controlled conditions involved the addition of Crone's nutrient solution and of an inoculum of the specific *Rhizobium* to the disinfected seeds at the time of planting. The inhibited plants were grown in sterilized soil and pots in the greenhouses of the department of agricultural bacteriology, University of Wisconsin, by Professor P. W. WILSON and co-workers. The *Melilotus* species were grown in the greenhouses of the department of genetics by Professor W. K. SMITH and those of *Medicago* by Professors R. A. BRINK and D. C. COOPER. The *Trifolium* series was obtained from plants grown by Dr. OLIVER SMITH of the U.S. Department of Agriculture. The plants, when nodules were at varying stages of maturity, were removed from the pots in which they were growing, and the exposed nodules and root tips were collected and fixed. Other material was obtained from plants of various species growing in the field. Chance inoculation had occurred in these, as well as in the plants of *Medicago*, *Melilotus*, and *Trifolium*.

Some material was fixed in Karpetschenko's modification of Nava-shin's fluid, imbedded in paraffin, and sectioned at thicknesses of 8-15  $\mu$ . Most of the nodules, however, were fixed in Carnoy's alcohol-acetic acid-chloroform solution and used for aceto-carmin smears. The latter method proved satisfactory for the determination of chromosome numbers in the nodules. Mitotic spindles in the meristematic region of the nodule are variously oriented. A higher percentage of polar views of equatorial plates is obtained by use of the smear technique than is possible in sectioned material. Either the apical meristematic portion of the nodule (fig. 1M) or the whole nodule, if in an early stage of development, was crushed in a drop of the stain and examined for division figures. This method was used

also for some root tips, but transverse sections of imbedded material usually gave a greater number of polar views of equatorial plates.

Although the somatic chromosome numbers for the majority of the species studied had previously been reported (47, 48, 49, 23, 24, 25, 26, 38, 44), a determination of the number was made in the root tips of each individual nodule-bearing plant.

Plants from which material used in this study was obtained may be classified in three groups: (1) thirty-one diploid species belonging to six genera (table 1); (2) plants belonging to three genera, each in-

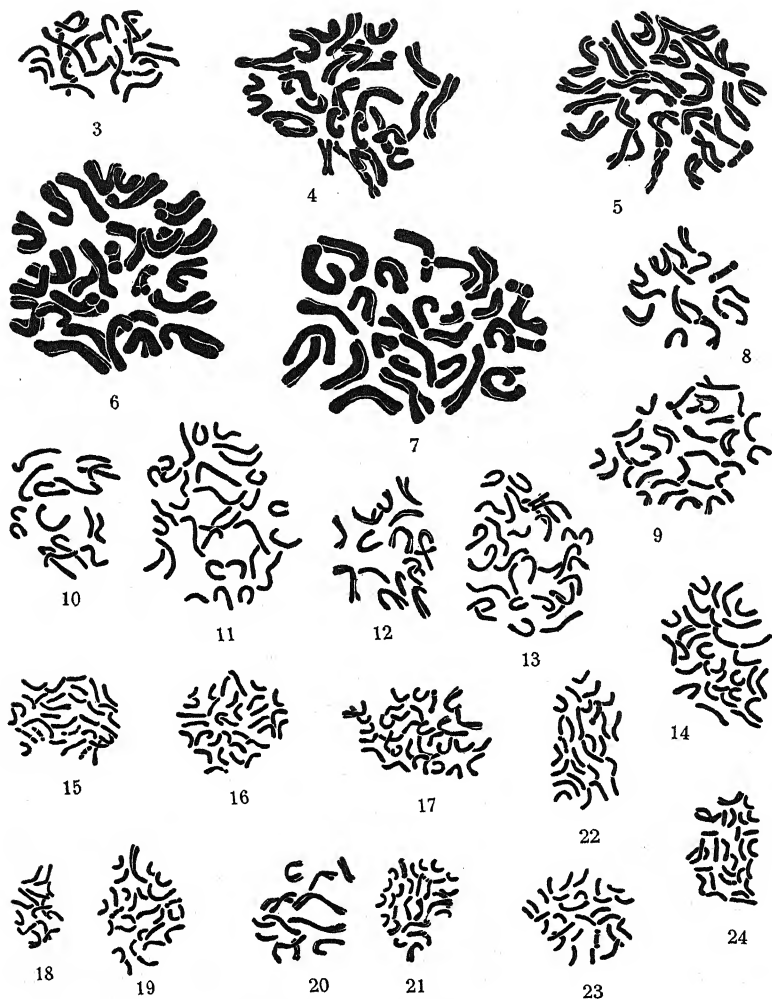


FIGS. 1, 2.—*Trifolium pratense*. Fig. 1, longsection through root nodule: *A*, older cells packed with bacteria; *B*, uninfected cortical cells; *M*, meristematic portion of infected area.  $\times 80$ . Fig. 2, polar views of equatorial plates: *M*, infected cell from meristematic region, 28 chromosomes; *B*, uninfected cortical cell, 14 chromosomes.  $\times 1580$ .

cluding a polyploid series (table 2); (3) plants known to be hindered in the fixation of free nitrogen by any of the means just mentioned (table 3).

### Observations

**DIPLOID SPECIES.**—The numbers of chromosomes on equatorial plates of dividing infected cells in root nodules of thirty-one leguminous species, including members of the genera *Lathyrus*, *Medicago*, *Melilotus*, *Pisum*, *Trifolium*, and *Vicia* (figs. 3–24), were determined and compared with the numbers present in root tips of the same plants (table 1). In each instance the chromosome number in the infected nodular cells was twice that present in root tip cells.



FIGS. 3-24.—Chromosomes (numbers in parentheses) from root tip cells and infected nodular cells of diploid plants. Fig. 3, *Vicia angustifolia*, nodular cell (24). Fig. 4, *Pisum sativum*, nodular cell (28). Fig. 5, *Lathyrus odoratus*, nodular cell (28). Fig. 6, *L. ochroleucus*, nodular cell (28). Fig. 7, *L. latifolius*, nodular cell (28). Fig. 8, *Melilotus altissima*, root tip cell (16). Fig. 9, *M. altissima*, nodular cell (32). Fig. 10, *M. caspia*, root tip cell (16). Fig. 11, *M. caspia*, nodular cell (32). Fig. 12, *M. gracilis*, root tip cell (16). Fig. 13, *M. gracilis*, nodular cell (32). Fig. 14, *M. alba* (diploid), nodular cell (32). Fig. 15, *Medicago arabica*, nodular cell (32). Fig. 16, *M. lupulina*, nodular cell (32). Fig. 17, *M. sp.*, nodular cell (32). Fig. 18, *Trifolium agrarium*, root tip cell (14). Fig. 19, *T. agrarium*, nodular cell (28). Fig. 20, *T. resupinatum*, root tip cell (14). Fig. 21, *T. resupinatum*, nodular cell (28). Fig. 22, *T. procumbens*, nodular cell (28). Fig. 23, *T. glomeratum*, nodular cell (28). Fig. 24, *T. incarnatum*, nodular cell (28).  $\times 1580$ .

TABLE 1  
CHROMOSOME NUMBERS IN DIPLOID SPECIES

SPECIES	NORMAL NUMBERS		INVESTIGATOR	NUMBER IN INFECTED CELLS
	n	2n		
ALFALFA CROSS-INOCULATION GROUP				
1. <i>Medicago arabica</i> All.....	8	16	Fryer (22), Chekhov (9)	32
2. <i>M. lupulina</i> L.....	8	16	Tischler (47)	32
		16	Fryer (22)	
			Ghimpu (27, 28), Chekhov (9)	
3. <i>M. platycarpa</i> Trautv.....	8		Tischler (47)	32
		16	Fryer (22)	
4. <i>M. ruthenica</i> Trautv.....		16	Fryer (22)	32
5. <i>M. sativa</i> L.....	16		Tischler (47), Kawakami (32)	64
	16	32	Reeves (42)	
		32	Elders (15), Fryer (22), Ghimpu (27, 28), Chekhov (9), Oppenheimer (41)	
6. <i>M. sp.</i> .....		16	Brink (unpublished) l.c.	32
7. <i>Melilotus alba</i> Desr.....	8		Cooper (12)	32
	8	16	Castetter (7, 8)	
		16	Elders (15), Fryer (22), Chekhov (9), Clark (11)	
8. <i>M. altissima</i> Thuill.....		16	l.c.	32
9. <i>M. caspia</i> Gruner.....		16	l.c.	32
10. <i>M. dentata</i> Pers.....		16	Chekhov (9)	32
11. <i>M. gracilis</i> DC.....		16	l.c.	32
12. <i>M. officinalis</i> Lam.....	8		Cooper (12)	32
		16	Elders (15), Fryer (22), Chekhov (9), Clarke (11)	
13. <i>M. segetalis</i> Ser.....		16	Clarke (10, 11)	32
14. <i>M. speciosa</i> Dur.....		16	Clarke (11)	32
15. <i>M. suaveolens</i> Ledeb.....		16	Chekhov (9)	32
16. <i>M. sulcata</i> Desf.....		16	Fryer (22), Chekhov (9), Clarke (10, 11)	32
17. <i>M. wolgica</i> Poir.....		16	Chekhov (9), Clarke (10)	32
CLOVER CROSS-INOCULATION GROUP				
18. <i>Trifolium agrarium</i> L.....		14	l.c.	28
19. <i>T. dubium</i> Sibth.....		28	l.c.	56
20. <i>T. glomeratum</i> L.....	7		Bleier (3)	28
		16	Wexelsen (50)	

TABLE 1—*Continued*

SPECIES	NORMAL NUMBERS		INVESTIGATOR	NUMBER IN INFECTED CELLS
	%	2%		
CLOVER CROSS-INOCULATION GROUP—Continued				
21. <i>T. incarnatum</i> L.....	8	.....	Bleier (3)	28
.....	.....	14	Karpetschenko (31),	
.....	.....	(14) 16	Wexelsen (50)	
22. <i>T. involucratum</i> Dulac.....	.....	28	Oppenheimer (41)	56
23. <i>T. pratense</i> L.....	7	.....	<i>l.c.</i>	28
.....	7	14	Kawakami (32)	
.....	.....	14	Bleier (3)	
.....	.....	14	Karpetschenko (31),	
.....	.....	14 (24)	Wexelsen (50)	
24. <i>T. procumbens</i> L.....	.....	14	Oppenheimer (41)	28
25. <i>T. repens</i> L.....	16	.....	Karpetschenko (31)	64
.....	14	.....	Kawakami (32)	
.....	.....	16	Bleier (3)	
.....	.....	16	Erith (16)	
.....	.....	32	Karpetschenko (31),	
.....	.....	(24) 28, 32	Wexelsen (50)	
26. <i>T. resupinatum</i> L.....	8	.....	Oppenheimer (41)	28
.....	.....	16	Bleier (3)	
.....	.....	14	Karpetschenko (31)	
.....	.....	14	<i>l.c.</i>	
PEA CROSS-INOCULATION GROUP				
27. <i>Lathyrus latifolius</i> L....	7	.....	Simonet (45)	28
.....	7	14	Winge (53), Fisk (17)	
28. <i>L. ochroleucus</i> Hook.....	.....	14	Senn (43)	28
29. <i>L. odoratus</i> L.....	7	14	Winge (53)	28
.....	7	.....	Kawakami (32), Simonet	
.....	.....	.....	(45)	
30. <i>Pisum sativum</i> L.....	7	14	Canon (6)	28
.....	7	.....	Winge (54)	
31. <i>Vicia angustifolia</i> L.....	.....	12	Sveshnikova (46)	24

Occasionally, in an aceto-carminic smear, diploid as well as tetraploid cells were found in the meristematic region of a nodule. In such a case, diploid cells occurred in a group in which the cytoplasm was clear and there was no evidence of infection. A cross section through the meristem of a pea nodule showed diploid cells in the uninfected cortical region. Tetraploid cells predominated in the central infected region.

No variation from the 2:1 chromosome relationship between infected and uninfected cells was found in any of the plants examined. Aberrant features of mitosis, such as absence of a spindle, multipolar spindles, lagging chromosomes, or irregularities in chromosome number, were not observed in any case.

**POLYPLOID SERIES.**—Polyploid series were available within the genera *Melilotus*, *Medicago*, and *Trifolium* (table 2). An autotetraploid strain of *Melilotus alba* ( $2n = 32$ ) had been obtained by ATWOOD (1) by means of heat treatment from the diploid *M. alba*

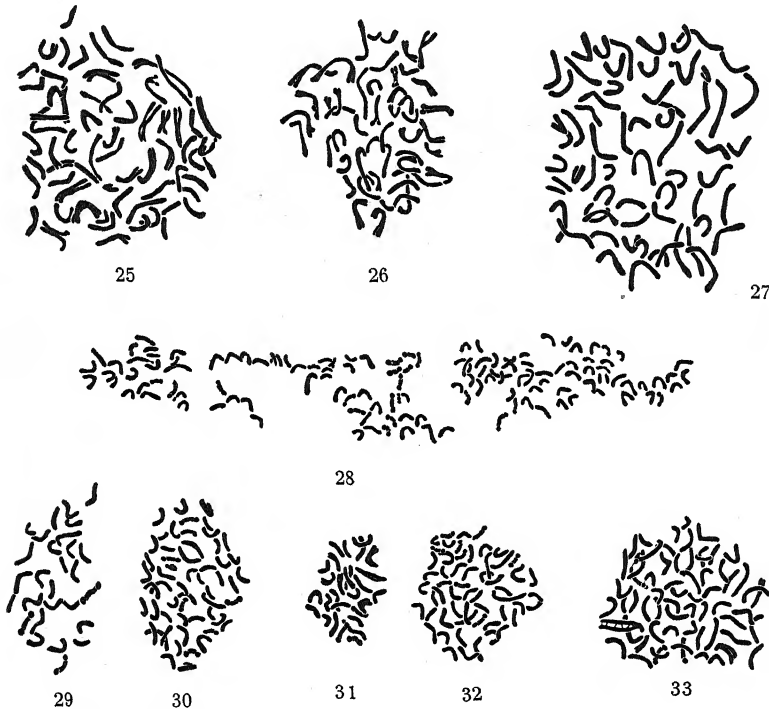
TABLE 2  
CHROMOSOME NUMBERS IN PLANTS OF POLYPLOID SERIES

SPECIES	NORMAL NUMBER ( $2n$ )	NUMBER IN INFECTED CELLS
Melilotus series		
alba (diploid).....	16	32
alba (tetraploid).....	32	64
Medicago series		
sp. (diploid).....	16	32
sativa (tetraploid).....	32	64
sativa $\times$ M. sp. (triploid).....	24	48
sativa (octoploid).....	64	128
Trifolium series		
agrarium.....	14	28
dubium.....	28	56
involutratum.....	28	56
repens.....	32	64

( $2n = 16$ ). The chromosome number (32) in the root tips of this plant, although already tetraploid, was exactly half the number (64) found in the infected cells of the nodule (figs. 14, 25).

An undetermined species of *Medicago*, with the basic chromosome number  $n = 8$  ( $2n = 16$ ), was found to have the tetraploid number (32) in the infected cells of the nodule (fig. 17). *Medicago sativa* is a natural tetraploid ( $2n = 32$ ) within this genus. The chromosome number in the infected cells of the nodule of this species is 64 (fig. 27). BRINK made a successful cross between *M. sativa* ( $2n = 32$ ) and the undetermined species ( $2n = 16$ ) just mentioned, and obtained a triploid plant with 24 chromosomes (unpublished). The number of chromosomes in the infected cells of the nodule of this triploid plant was found to be 48 (fig. 26). An artificially induced autotetraploid alfalfa with 64 chromosomes (octoploid in terms of

the basic number for the genus) has been obtained by COOPER (13). One hundred twenty-eight chromosomes are present in the infected cells of the nodule of this plant. An anaphase configuration in an infected cell (fig. 34) shows two groups, each with 128 chromosomes,



FIGS. 25-33.—Chromosomes from root tip cells and infected nodular cells of polyploid plants. Fig. 25, tetraploid *Melilotus alba*, nodular cell (64). Fig. 26, triploid plant, cross between *Medicago sativa* and *M. sp.*, nodular cell (48). Fig. 27, *M. sativa*, nodular cell (64). Fig. 28, tetraploid *M. sativa*, nodular cell (128).  $\times 1080$ . Fig. 29, *Trifolium dubium*, root tip cell (28). Fig. 30, *T. dubium*, nodular cell (56). Fig. 31, *T. involucreatum*, root tip cell (28). Fig. 32, *T. involucreatum*, nodular cell (56). Fig. 33, *T. repens*, nodular cell (64).  $\times 1580$ .

on the spindle. Figure 28 shows one of these groups in which the chromosomes were so arranged that the number could be rather readily determined.

A natural polyploid series exists in *Trifolium*. Six of the nine species examined (table 1) show 14 chromosomes in root tip cells;

two—*T. dubium* Sibth. (fig. 29) and *T. involucreatum* Dulac (fig. 31)—possess 28, and one (*T. repens* L.) has 32. Here again the chromosome number in the infected cells of a nodule is in each case twice the number present in root tips of the same plant (figs. 18–24, 29–33).

PLANTS WITH INHIBITED NITROGEN FIXATION.—Some *Rhizobium* strains (designated as “poor” or “ineffective”) become parasitic

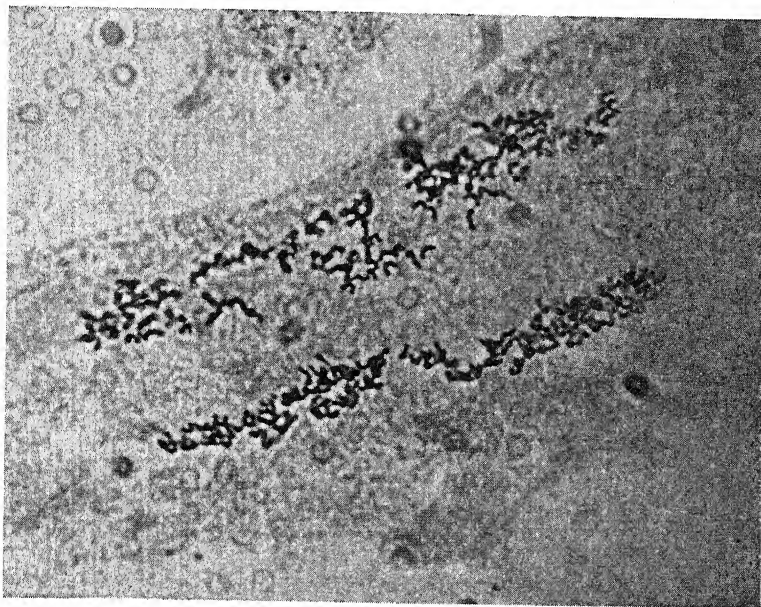


FIG. 34.—Anaphase in nodule of tetraploid *Medicago sativa*; 128 chromosomes (16n) passing to each pole; upper chromosome group shown in fig. 28.

rather than symbiotic upon the host plant (18, 21). When a plant is infected by a poor strain, an abundance of nodules may be formed scattered over the entire root system but fixation of free nitrogen does not occur. In nodules of *Pisum sativum* L. formed in consequence of inoculation with University of Wisconsin strain 311 (ineffective) of *Rhizobium leguminosarum* Frank, the chromosome complement of the infected cells was, as in previous cases, double the number in normal cells (table 3, fig. 35).

Seedlings of *Pisum sativum* were inoculated with an effective strain of *Rhizobium leguminosarum* (U. W. strain 317). A solution of

calcium nitrate was added to the soil in which the plants were growing at the time of inoculation and again each week thereafter for five weeks. When combined nitrogen is added to the nutrient, very few nodules are formed and the fixation process is inhibited (21, 29). The nodules produced after this treatment grew slowly, were few and small. Sampling was difficult because of the reduced number of nodules available, and mitotic figures were less numerous than in more rapidly growing nodules. The number of chromosomes present in the infected cells (fig. 36), however, was double that found in root tip cells.

TABLE 3  
CHROMOSOME NUMBERS IN PLANTS WHOSE NITROGEN  
FIXATION IS INHIBITED

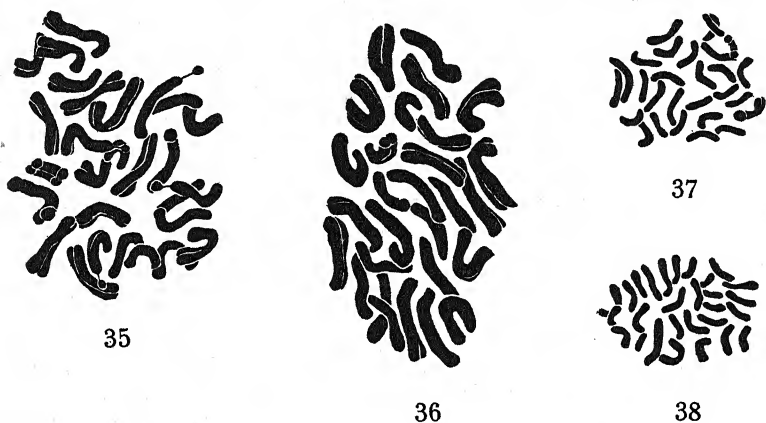
SPECIES	NORMAL NUMBER (2n)	NUMBER IN INFECTED CELLS
Ineffective strain of <i>Rhizobium</i>		
<i>Pisum sativum</i> inoculated U.W. strain 311	14	28
Nitrate ( $\text{CaNO}_3$ ) added to nutrient		
<i>P. sativum</i> inoculated U.W. strain 317...	14	28
Hydrogen		
<i>Trifolium pratense</i> ( $\text{Al} + \text{NaOH H}_2$ ).....	14	28
Carbon monoxide		
<i>T. pratense</i> (air + 0.1% CO).....	14	28

WILSON and UMBREIT (52) found that if hydrogen is supplied to the atmosphere surrounding the host plant, the fixation process is inhibited. In the experiments with which the present paper is concerned, the germinated seedlings of *Trifolium pratense* L. were inoculated at the time of planting. The plants were allowed to grow for three or four weeks, or until the second or third trifoliate leaf appeared, then hydrogen was substituted to a large extent for the air in the bottles in which they were growing. Once a week the plants were aerated and the gas was changed. Carbon dioxide was added when necessary. The chromosome number in the nodules under these conditions was the same as before treatment ( $28 = 4n$ , fig. 37).

Carbon monoxide, when added in like manner, similarly inhibits the fixation of nitrogen (private communication from P. W. WILSON and S. B. LEE). This gas was supplied to red clover seedlings. The

plants so treated showed a great decrease in nitrogen fixation. The infected cells from the nodules, however, were tetraploid ( $28 = 4n$ , fig. 38).

**CHROMOSOME CHARACTERS.**—Equatorial plates in infected cells (figs. 9, 11, 13) are larger, and the chromosomes are more compactly arranged, than in normal diploid cells (figs. 8, 10, 12). The individual chromosomes in infected cells closely resemble in form and size those in cells of root tips. A marked similarity can be noted be-



FIGS. 35-38.—Chromosomes from infected nodular cells of plants whose fixation of atmospheric nitrogen had been inhibited. Fig. 35, *Pisum sativum* after inoculation with ineffective strain of *Rhizobium* (U.W. strain 311); 28 chromosomes. Fig. 36, *P. sativum*,  $\text{CaNO}_3$  added to nutrient (28). Fig. 37, *Trifolium pratense* grown in atmosphere containing added hydrogen (28). Fig. 38, *T. pratense* grown in atmosphere containing added carbon monoxide (28).  $\times 1580$ .

tween the chromosomes on a normal somatic equatorial plate (fig. 8) and those on an equatorial plate in an infected cell (fig. 9) of the same species. In those instances in which a pair of chromosomes in a somatic equatorial plate can be identified with certainty because of some particular morphological feature, four similar chromosomes appear in equatorial plates in infected cells of a nodule on the same plant. An infected cell of *Pisum sativum* inoculated with an ineffective strain (fig. 35) shows four similar satellite chromosomes, whereas two such chromosomes are present in root tip cells. Although similar comparisons for each individual chromosome have not been

attempted, size, shape, and general appearance indicate that the complement of an infected cell results from a simple doubling of the normal complement.

The species whose normal chromosome numbers are here first reported are:

<i>Melilotus altissima</i> .....	$2n=16$ , fig. 8
<i>M. caspia</i> .....	$2n=16$ , fig. 10
<i>M. gracilis</i> .....	$2n=16$ , fig. 12
<i>Trifolium agrarium</i> .....	$2n=14$ , fig. 18
<i>T. dubium</i> .....	$2n=28$ , fig. 29
<i>T. involucreatum</i> .....	$2n=28$ , fig. 31

### Discussion

It appears from the results that the chromosome number in the infected cells of nodules of every plant studied is twice the number present in the uninfected root tip cells of the host. The same relationship exists in plants so treated as to inhibit the fixation of free nitrogen. No irregular mitotic phenomenon has been observed in any case.

The observations on diploid plants (those possessing twice the basic number for the genus) might seem to indicate that a certain chromosome number is necessary for this symbiotic relationship. When the polyploid series are considered, however, it appears that the absolute chromosome number is not the significant factor, but rather the 2:1 relationship between infected and uninfected tissue. It appears, too, that this relationship is not disturbed once the nodules are initiated, even though nitrogen fixation is partially or totally prevented. A study of the development of the nodule beginning with the cell or cells first infected, involving the recognition of the initiation of polyploidy and an observation of the multiplication of polyploid cells, may be expected to suggest the causal factor or factors concerned.

It has long been known that nuclear size and cell size are commonly, though not invariably, correlated with chromosome number (4). Meristematic infected nodular cells have approximately twice the volume of the uninfected (diploid) cells of the nodular cortex. As the infected cells of a young nodule mature, they become more or less gorged with rhizobia (fig. 1A). The nuclei of these mature host cells are in varying stages of disintegration.

Infected overgrowths of various types have been found to possess chromosome numbers differing from those typical for the species. Diploid, tetraploid, and octoploid cells have been observed in crown gall tissue of sugar beet (55) and of beet and tobacco (36, 34). Wide variation in chromosome numbers, ranging from haploid to tetraploid or even higher, are found in the carcinomatous tissue in the fowl, rat, and man (36). Abnormal tissues, such as the galls produced by parasites, contain tetraploid cells (40). Gall mites upset the meiotic divisions in *Lycium halimifolium*, resulting in many irregularities in the chromosome numbers (33).

Agents, including various chemical substances, heat, cold, x-ray, and wounding, have been used successfully to produce polyploidy in a number of forms. BLAKESLEE and AVERY (2) obtained plants with irregularly increased chromosome numbers by the application of colchicine to the seeds or seedlings. Buds from callous tissue in tomato and *Solanum* spp. formed tetraploid shoots (30, 37). It is possible either that tetraploidy was produced by the wounding, or that tetraploid cells were already present. WHITAKER (51) reports occasional tetraploidy in cells of nonparasitic tumors on grafted tobacco. WULFF (57), as well as earlier workers, has found "polysomaty" occurring naturally in the periblem of several genera of the Chenopodiaceae. This condition is reported also in *Cannabis sativa* (5). A preliminary examination of dividing cells in the root hair region of *Pisum sativum* showed a few scattered tetraploid figures in the cortex near the endodermis. The chromosomal condition in leguminous root nodules differs, so far as is now known, from that in other stimulated growths by the constant occurrence of the doubled number. It is not the mere doubling that seems to be most significant, however, rather is it the 2:1 chromosome balance that is established between infected and uninfected cells. This 2:1 ratio is so constant that it may well have some definite bearing upon the symbiotic relationship between the host plant and the infecting organism.

### Summary

1. Infected cells of root nodules of thirty-one diploid species belonging to *Lathyrus*, *Medicago*, *Melilotus*, *Pisum*, *Trifolium*, and *Vicia* contain in each case the tetraploid number of chromosomes.

2. Uninfected cells in the nodular cortex possess the diploid number characteristic of the particular species.

3. The chromosome number in infected nodular cells in diploid and autotetraploid strains of *Melilotus alba* is in each case twice that characteristic of uninfected somatic cells of the same plant.

4. The chromosome number in infected cells of the nodules from diploid, triploid, tetraploid, and octoploid species and races of *Medicago* is in each case double that in root tip cells.

5. Infected nodular cells from plants of a natural polyploid series in *Trifolium* possess in each case twice the somatic number of chromosomes.

6. Inhibition of the fixation of free nitrogen by such conditions as infection with an ineffective strain of *Rhizobium*, the presence of combined nitrogen in the nutrient, or the presence of hydrogen or of carbon monoxide does not affect the chromosome numbers of the nodular cells. Infected cells regularly present twice the number found in uninfected cells.

7. There is definitely and consistently a 2:1 chromosome ratio between infected and uninfected cells of the root nodules on the leguminous plants examined.

8. Diploid chromosome numbers herein reported for the first time are: *Melilotus altissima*  $2n = 16$ , *M. caspia*  $2n = 16$ , *M. gracilis*  $2n = 16$ , *Trifolium agrarium*  $2n = 14$ , *T. dubium*  $2n = 28$ , and *T. involucreatum*  $2n = 28$ .

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# PHYSIOLOGY OF LAZY CORN

JOHN SHAFER, JR.<sup>1</sup>

(WITH THREE FIGURES)

## Introduction

One recessive gene, showing typical Mendelian inheritance, controls the laziness of lazy corn. If that gene is present in homozygous condition it causes the plant to be lazy. Under field conditions lazy corn appears to be normal at first; it grows erect. At some time during its development (in these experiments, when it has reached a height of 1 to 2 feet) it begins to lean from the vertical, and this leaning becomes more and more pronounced until the plant is lying on the ground. The bending appears to occur entirely in the first two or three internodes above the ground; the roots remain firmly fixed in the soil. The plant continues growth along the ground with no apparent tendency to become erect (save in rare cases). Finally, it produces tassel and ear in the manner of ordinary corn.

VAN OVERBEEK (10) has shown that during the first few days of their development, lazy plants are normal in respect to geotropism. Then they lose the ability to respond and become ageotropic. Growth measurements of stems of partly mature corn plants, lazy and normal, indicate that the difference between the two sorts is at least partly one of growth, although JENKINS (5) has shown that laziness is probably due in part to mechanical weakness. Since growth differences between lazy and normal corn were evident, it was hoped that some abnormality in the amount or distribution of the growth hormone would be found to explain the abnormal geotropic response of lazy corn.

## Experimentation

### GROWTH MEASUREMENTS

Numerous measurements were made of the growth of stems of nearly mature corn. A number of evenly spaced sewing needles were

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soldered to a piece of brass, forming a "comb" with which a stem could be marked by pricking through the inclosing leaf sheaths (4). The distance between the original needle marks being established, final measurements would disclose how much growth had taken place between the marks and in what region of the original stem it had occurred. The distance between needles averaged 0.76 mm. The holes produced by such needle pricks were apparent even after two weeks, except in very young, rapidly growing tissues. The final distances were measured with an ocular micrometer in a dissecting microscope, and were read to the nearest 0.05 mm. All marks for growth measurements were made at and just above nodal regions, and measurements were made from the external upper point of leaf insertion on the stem as the zero point, or node.

In both lazy and normal plants the growing zone was found to be 1 to 4 mm. above the insertion of the leaf (fig. 1). The growing zone varied from 0 to 10 mm. in length, with the average length of those measured about 3 mm. In the longer growing zones the upper parts had grown relatively slowly, however, most of the growth being in a zone of only a few millimeters. In very young nodal regions the growing zones were wider than in the older ones, and in the former growth was exceedingly rapid.

It was easy to induce growth in nodes of normal plants which would not ordinarily have grown more (fig. 2A, C). It was necessary only to place the plants horizontally, and nodes one or more internodes below those which grew in control plants would show distinct growth. This inauguration of growth in mature corn nodes agrees with the findings of earlier workers (8, 9, 6). They have stated that nodes of various grasses which have stopped growth begin to grow again when placed horizontally. SACHS reported considerable shortening of the upper sides of the up-bending nodes, whereas JOST found little or no shortening. The present work indicates that both conditions exist, even in the same plant (fig. 2A). JOST showed further that the sides of maize stems grow an amount equal roughly to the average of top and bottom growth. This, too, was confirmed by a few measurements made during the present study. The induced growth, occurring mainly in the lower side of the stem, caused the plants to bend upward. The new tissue formed a visible wedge, dis-

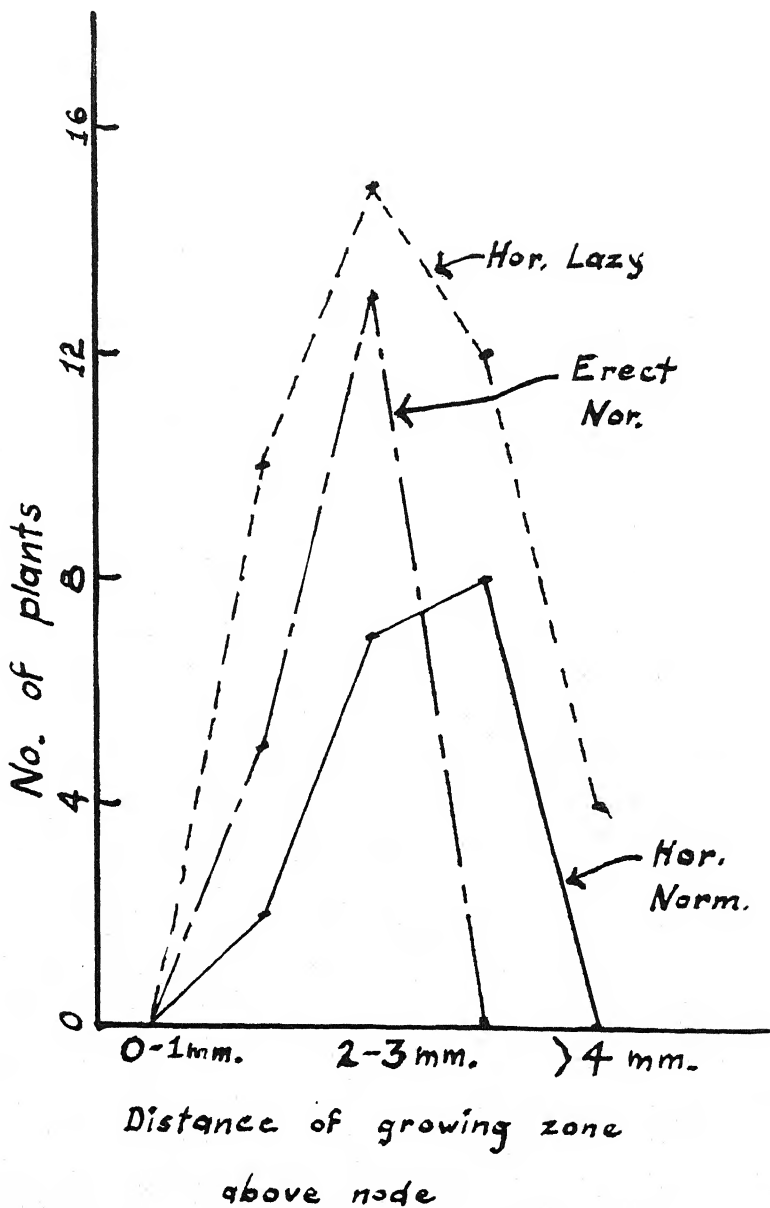


FIG. 1.—Frequency curve showing distances of lower edges of growing zones above their nodes. Curves are for: horizontal lazy; horizontal normal; and erect normal plants.

tinct in appearance from the ordinary stem tissue, and having its broad edge downward.

Microscopic examination of a few hand sections of this new tissue, kindly prepared by Mr. M. W. QUIMBY, indicated that the epidermal cells enlarge, as SACHS (9) states; that the parenchyma

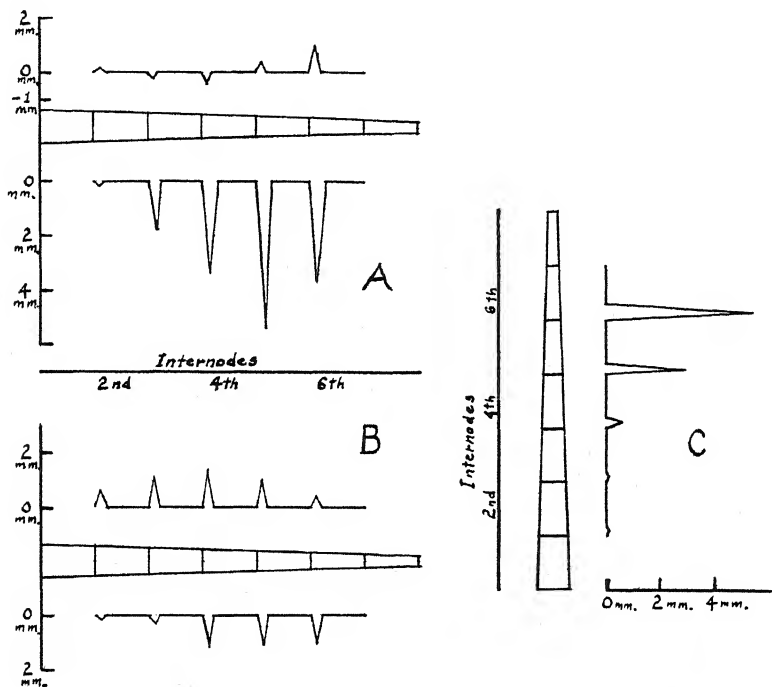


FIG. 2.—Schematic representation of growth of corn stems (shown with only seven internodes). Average amount of growth of each internode shown in mm. (on ordinate in A and B; on abscissa in C). A, horizontal normal; B, horizontal lazy; C, erect normal.

cells of the pith do not enlarge (SACHS states that they do) and must therefore multiply; that the newly formed vascular strands are much larger than normal, owing to the presence of many sclerenchymatous fibers.

Several wedges of new tissue were found at the bases of prostrate lazy plants, but with the broad edge up (as if the lazy plant had grown down). A horizontal position had no effect on the growth of

lazy corn, so far as could be determined (fig. 2*B*); one would expect this from the lack of response of lazy to gravity. There was some indication that lazy nodes continued growing after comparable normal ones had ceased growth (fig. 2*B*, *C*).

In some cases the holes on the upper side of horizontal normal plants were closer together after the plants had bent up than were the original needle pricks, showing that growth of the bottom side had actually compressed the top side (fig. 2*A*). In some such cases the top side appeared wrinkled. More often there was no sign of compression of the top side of horizontal normal plants, the stems having remained about the same size or even grown a little.

No method was found to induce growth in recently mature lazy nodes. The external application of 0.2 per cent heteroauxin-lanolin paste to such nodes had absolutely no effect. On normals such paste occasionally caused slight growth in thickness, but never any measurable growth in length.

#### AUXIN PRODUCTION

There proved to be no obvious correlation between the amount of auxin obtained by diffusion from a coleoptile and the normal or lazy nature of the older plant. Seeds of such genetic nature that they would produce about 50 per cent lazy and 50 per cent normal plants were germinated in the dark. The coleoptile tips were cut off when 1-2 cm. above the ground, and were placed on small agar blocks, one tip to a block. After time had been allowed for the outward diffusion of the auxin, the blocks were tested for auxin content by the standard *Avena* test (12). The yield of auxin varied considerably, probably depending in part on the age and height of the plants from which the coleoptiles were cut. After removal of the coleoptiles, the plants were placed in the greenhouse to grow until they were old enough for the lazies to be ageotropic. Then they were tested by being placed horizontally (10); finally, the amount of bending of each was compared with the auxin yield of its coleoptile. The average yield of auxin by coleoptile tips of lazy plants was slightly higher than that by tips of normal plants.

For each of the experiments on the production of auxin by coleoptile tips, the correlation coefficient has been calculated. Nine

such experiments were performed; of these, eight showed so little correlation between auxin yield of tips and laziness of older plants that the odds in favor of the significance of such correlation were well below 9:1. In one case the odds in favor of significance were greater than 19:1. In view of the eight experiments showing no correlation, this one case of rather definite correlation must have been due to chance. It is concluded that there is no correlation between auxin yield of coleoptile tips and laziness of older plants. Since VAN OVERBEEK (10) reported young seedlings of lazy corn to be normally geotropic, this is the expected result.

Efforts to obtain auxin by diffusion, in a manner similar to that mentioned in the preceding paragraph, failed when applied to first internodes (mesocotyls) of two-weeks-old seedlings, to stems and leaf bases of such seedlings, and to stem sections of nearly mature plants. Either there is little auxin in such tissues or the destruction at the cut surfaces is high. The presence in the auxin-receiving agar blocks of various substances had no effect; sucrose, cysteine, ascorbic acid, and indole(3)propionic acid were tried.

#### AUXIN TRANSPORT

Some transport tests gave instructive although not conclusive results. Transport of auxin through first internodes was never found. Even concentrations of auxin equivalent to  $450^\circ$  oat curvature, placed on top of an internode piece 10 mm. long for 110 minutes, or  $675^\circ$  auxin on an internode piece 4 mm. long for 60 minutes, were not sufficient to produce apparent transport. That is, no measurable amount of auxin moved from a block of agar containing auxin of such a concentration and placed on top of the internode section, through the section and into the block of plain agar on which the section was standing.

Sometimes transport was obtained through tender growing zones of stems of semi-mature plants. Probably the condition determining whether or not there would be such transport was the stage of development of the section of tissue used, but that is not certain. In some transport experiments there were used vertical sections of stems, or slabs cut from stems and placed horizontally; these types of tests were abandoned in favor of a combination type. In the

preparation of sections for all the transport experiments, care was taken to select similar appearing plants and to choose from them sections that were apparently comparable.

For the combination type of test, sections 1 cm. long of growing zones were cut and clamped firmly in a horizontal position. The erstwhile bottom end was divided into two equal parts by the horizontal insertion of a piece of razor blade. Above and below this were placed plain agar blocks, each resting flat against its half of the cut surface. On the top end of the section was placed agar containing auxin. The concentration of this auxin was varied in the direction that seemed to promise the best transport results. From such an experiment some measure of the lateral transport is obtained by the relative amounts of auxin in the upper and lower agar blocks on the basal end; likewise the total transport through the stem section is determinable.

In general these tests showed greater transport by lazy than by normal stems (table 1 and figure 3). Transport by the lazy was not always greater than that by the normal which had been selected as a check; this might be expected from the impossibility of getting uniform, comparable tissue. The plants used were nearly always from seeds from the same ear; but since this seed was not inbred, the plants must have varied much in spite of the care used to select similar ones. At times there was no appreciable transport; occasionally normal transported more than lazy; usually lazy transported the more—nearly twice as much, on the average (table 1 and figure 3).

As for the lateral transport determined from these same experiments (table 1), normal stem sections showed more auxin in the lower agar block six times, more in the upper one twice, and essentially the same amount in each block nine times; of these nine cases, six were due to insignificantly small transport. The ratio of auxin in the lower blocks to that in the upper was about 3:2. Lazy, out of seventeen experiments, showed more auxin in the lower block five times, more in the upper one seven times, and the same amount in each five times, of which two were due to lack of transport. The ratio of bottom to top was about 4:5.

The greater amounts of auxin in the lower blocks of normal corn

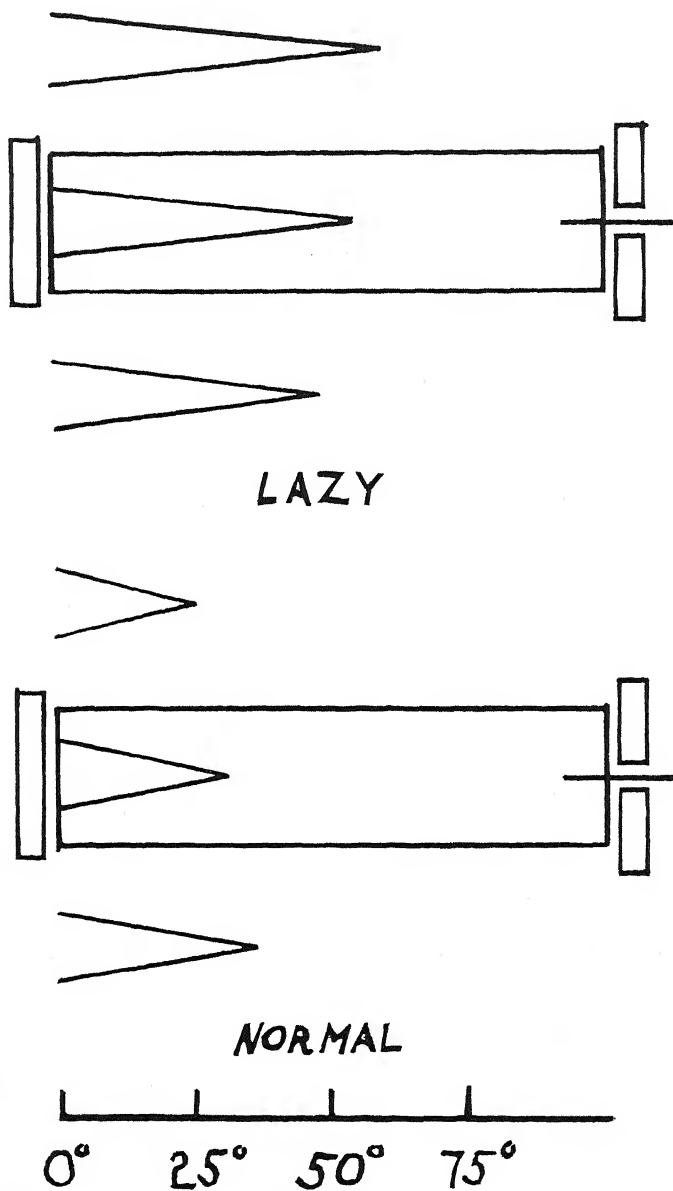
is to be expected from similar work on other plants. Several investigators (3, 7, 2, 1) have reported that in horizontally placed, negatively geotropic plant parts auxin becomes more concentrated in the lower side. The ratio of auxin in lower halves of plant parts to that in upper halves is reported variously as between 54:46 and

TABLE 1  
AUXIN TRANSPORT BY GROWING ZONES OF SEMI-MATURE  
LAZY AND NORMAL PLANTS

EXPERIMENT NO.	LAZY*			NORMAL*		
	TOP	BOTTOM	AVERAGE	TOP	BOTTOM	AVERAGE
1.....	4.3	4.3	4.3	0.5	0.3	0.4
2.....	3.3	5.8	4.6	— 0.3	— 0.2	— 0.25
3.....	1.8	— 0.3	0.75	4.3	4.6	4.5
4.....	6.0	2.5	4.25	1.2	2.5	1.8
5.....	7.2	3.2	5.25	1.5	1.4	1.5
6.....	3.3	3.0	3.2	3.8	2.2	3.0
7.....	2.5	3.5	3.0	2.2	4.5	3.3
8.....	1.4	4.8	3.1	1.7	1.5	1.6
9.....	4.3	1.3	2.8	1.5	2.7	2.0
10.....	1.5	2.2	1.8	0.2	0.4	0.3
11.....	0.4	0.2	0.3	— 1.0	0.2	— 0.4
12.....	16.2	9.0	12.6	0.25	1.3	0.75
13.....	2.0	2.3	2.1	1.4	— 0.3	0.5
14.....	— 0.2	0.0	— 0.1	0.1	0.2	0.1
15.....	3.2	7.3	5.2	— 0.4	— 0.9	— 0.7
16.....	0.8	0.0	0.4	4.1	7.1	5.9
17.....	1.8	— 0.3	0.75	4.3	8.9	6.6
Total...	59.8	48.8	54.3	25.4	36.4	30.9

\* Nearly all these figures are the average of curvatures of six or twelve test plants.

75:25. The ratio just reported for sections of normal corn stem falls within this range (*ca.* 60:40). The data in table 1 show that there is no downward transport of auxin as it passes through the horizontal sections of lazy stem. On the contrary they suggest that there may be some upward transport. This in turn suggests that semi-mature lazy plants are positively geotropic. The data are insufficient to prove this, but VAN OVERBEEK (11) has recently obtained corroborative evidence.



*Amount of auxin transported*

FIG. 3.—Schematic representation of auxin transport through sections of corn stems. Diagrams are idealized drawings of experimental set-up. Amount of auxin transported shown by heights of wedges. Transport through top and bottom halves of sections, and average of these, shown. Transport given as total degrees of oat curvature (see table 1).

## TEMPERATURE AND LAZINESS

Efforts to study the effects on laziness of light intensity and duration failed because of temperature variations; consequently temperature effects were investigated. Three chambers were prepared, of which one was kept at about  $10^{\circ}$  C. (range  $8.5^{\circ}$  to  $10.5^{\circ}$ ); one at about  $22^{\circ}$  C. ( $21^{\circ}$  to  $24^{\circ}$ ); the third at about  $30^{\circ}$  C. ( $29^{\circ}$  to  $33^{\circ}$ , but dropped twice to  $23^{\circ}$  for periods of a few hours). The light in each chamber was artificial, coming from a 150 watt Mazda bulb about 1.5 feet from the plants. The light intensity was low but was nearly the same in all three chambers, having been adjusted with the aid of a Weston light-meter. Since the light came from the side and the plants were all bent sideways, phototropic response offered the most simple test for laziness, requiring only that the plants be turned at the start of a test to be bending away from the light. Such a test, depending on the amount of bending back toward the light, seemed as satisfactory as VAN OVERBEEK's geotropic test (10). To be sure, it is not certain that lazy plants become aphototropic and ageotropic at the same time. However, the plants in these tests were aphototropic when about of a size which should have been ageotropic. Moreover, VAN OVERBEEK's geotropic test for lazy would not work in ordinary light unless the plants were both ageotropic and aphototropic.<sup>2</sup> In these temperature experiments both lazy and normal plants from 100 per cent lazy and normal lots of seed of different strains were tested. Although the temperature control was not exceedingly exact, yet the results are fairly distinct and convincing.

In general, the higher the temperature the earlier the lazy plants became lazy. When grown at  $30^{\circ}$  C. they became lazy about three days after germination. At  $22^{\circ}$  it took three and one-half to four days for laziness to appear. At  $10^{\circ}$  the plants remained partly normal until eight or ten days after germination. All lots became lazy when they were of about the same height, regardless of the number of days between germination and the attainment of that

<sup>2</sup> Tests recently carried out with twenty-three horizontal lazy seedlings and as many erect ones of the same age with unilateral light showed only a few of the plants beginning to bend up in three days; also, a few were beginning to bend toward the light. This further indicates that lazies become aphototropic and ageotropic at about the same time.

height. Apparently temperature, per se, does not affect laziness; the effect is through an influence on the growth rate. It would seem that a lazy plant becomes ageotropic and aphototropic at a rather definite stage of development.

### Discussion

Growth measurements of semi-mature lazy and normal corn stems (*Zea mays* L.) showed that both forms grow only in definite regions of the stem, which regions begin 1-4 mm. above the insertion point of the leaf. The growing regions vary in length from nothing to about 1 cm., being longer in the younger, more rapidly growing parts of a stem. The growth of normal stems is strongly influenced by gravity, even to the extent of recommencing if it has ceased not too long since. In such cases of induced growth it is the under side which grows, the upper side remaining the same length as before, or in some cases being compressed. The growth of lazy stems seems unaffected by gravity, although there is some indication that it persists longer than in normals.

The production of auxin by coleoptile tips bears no relationship to the lazy or normal nature of mature plants. Since seedling plants of lazy show normal geotropism, this is to be expected.

Transport tests in which sections of semi-mature stems were used suggest that lazy transports more auxin than normal corn. Of more importance is the lateral distribution of this auxin. In normal corn more of it is transported in the lower side of the stem, the ratio of bottom to top being about 3:2. This agrees with the findings of other workers in regard to other negatively geotropic plant parts. On the other hand, the auxin in lazy stems does not accumulate in the bottom of the stem but seems to move toward the top side, the ratio of bottom to top being 4:5. This suggests that lazy corn is positively geotropic.

Temperature studies, in which phototropism was used as a test for lazy, indicate that the time of appearance of laziness is definitely affected by temperature. The higher the temperature the shorter the time between the germination of the seed and the appearance of laziness. Regardless of temperature, however, the plants become lazy when of a fairly definite size. Hence it seems probable that they

become lazy at a definite stage of growth, and that temperature affects laziness indirectly, through its effect on the growth rate.

### Summary

1. Corn stems grow only in definite intercalary regions which are 1 to 4 mm. above the leaf insertion points, and are 0 to 1 cm. long.
2. Growth can be induced in recently mature normal nodes by placing the plants in a horizontal position. Such a position has no effect on the growth of lazy stems.
3. Lazy stems tend to grow for a longer time than do normal ones.
4. Auxin yield of coleoptile tips is independent of laziness.
5. Auxin is transported more readily by lazy than by normal stems.
6. Auxin is so redistributed in horizontal normal stems that about 60 per cent of it moves to the lower side. This redistribution is reversed in lazy stems, so that about 55 per cent moves in the upper half.
7. Low temperature delays the time of appearance of laziness, but probably only by slowing up the growth rate.

This work was done at the California Institute of Technology. The writer is indebted to the National Research Council for the grant making the study possible, and to the members of the staff at the Institute for their help.

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# LOCALIZATION OF PHOTOPERIODIC PERCEPTION IN *HELIANTHUS TUBEROSUS*<sup>1</sup>

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 502

KARL C. HAMNER AND ELBERT M. LONG

(WITH FOUR FIGURES)

## Introduction

Among the many papers on photoperiodism published within the last few years, convincing evidence for photoperiodic perception by leaves in a wide range of plants has been presented (1-9).

On the basis of experiments with the Jerusalem artichoke, *Helianthus tuberosus* L., ZIMMERMAN and HITCHCOCK (11) found that tubers were formed by the plants maintained continuously on conditions of short day, and by those capped with the black bags, but none were formed on the plants exposed to the long days of summer. They describe the three sets of conditions as follows: "Under normal day length during summer months; plants covered with a black cloth from 4:30 P.M. to 9 A.M.; stem tips, only, capped with black cloth from 4:30 P.M. to 9 A.M." They concluded that "there is every indication that the growing stem tip has a regulatory influence on the development of underground stems and tubers. It seems likely that the regulators are chemical agents of a hormone-like nature, manufactured in the stem tip and sent to other parts of the plant where they can exert a controlling influence on the development of the underground portions."

Because of the amount of recent evidence, including that of FABIAN (3) dealing with tuber formation in *Ullucus tuberosus*, nearly all of which indicates that the leaves are the principal organs of photoperiodic perception, the following experiments were conducted to investigate the behavior of the Jerusalem artichoke.

<sup>1</sup> This investigation was aided in part by a grant to the University of Chicago from the Rockefeller Foundation.

### Methods

One hundred tubers of *Helianthus tuberosus* L., each weighing about 50 gm., were selected for uniformity and planted singly in 8-inch pots in light garden soil. The soil was kept moist, and as soon as the tip of the first shoot appeared, all the pots were transferred to a greenhouse bench provided with supplementary illumination of Mazda light at an intensity at the soil surface of about 100 foot candles, from sundown until 2:00 A.M. The duration of the photoperiod on this bench (referred to as the long photoperiod bench) varied somewhat, but was never less than 18 hours. During their first month of growth, all the plants were fertilized by watering twice weekly with a modified, double strength, SHIVE's (10)  $R_2S_5$  nutrient solution, to supplement the nutrients already present in the soil.

On April 18, when the plants were a little over two weeks old, they were segregated for various treatments. Six pots were transferred to a short photoperiod bench, which was covered regularly, except from 7:30 A.M. to 4:30 P.M., with a curtain of two layers of black sateen cloth, thus providing photoperiods of 9 hours and dark periods of 15 hours' duration. Another group of six pots was left on the long photoperiod bench, but one leaf was given short photoperiod by the use of small cylindrical cardboard boxes as described previously by HAMNER and BONNER (4). The terminal bud and young leaves of another group of six plants were given short photoperiod by an adaptation of the same method, as shown in figure 1. Cardboard boxes were placed in position over the tip of the plant at 5 P.M. and removed at 8 A.M. The boxes were about 3 inches in length, and expanding leaves which were less than 3 inches in length were included in the treatment. The stems were strong enough to support the weight of the lid of the box. A mixture of clay flour and castor oil was used to seal the space between the stem and the lid. The boxes were suspended in position by strings made fast overhead.

In a third group of six plants, only the terminal bud was placed on short photoperiod. Before the box covers were put in place all the young leaves were trimmed off with a sharp scalpel, except those 5 mm. or less in length. As new leaves expanded, they too were trimmed off, usually before they reached lengths of 1 cm. In order to make sure that this somewhat drastic treatment did not in itself

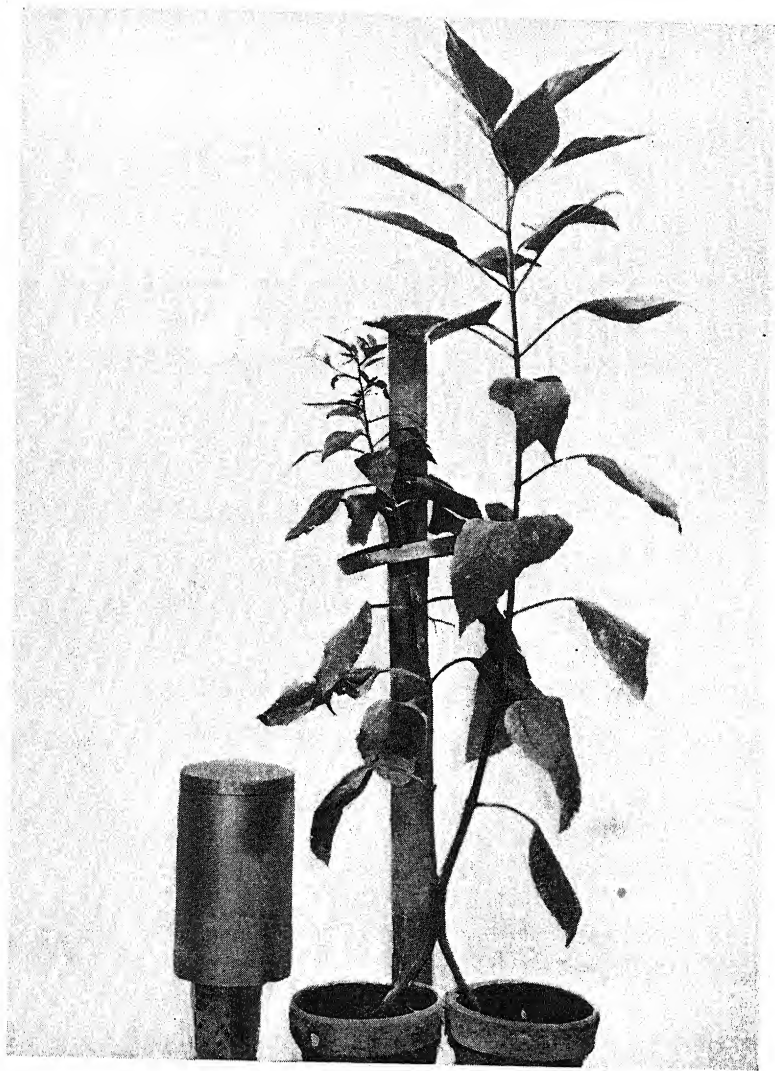


FIG. 1.—Method by which stem tip and leaves up to 2 inches in length were subjected to short photoperiod while remainder of plant was on long photoperiod. Box in position at left, detached at right.

induce nor inhibit tuber formation, the young leaves of twelve other plants were similarly trimmed; six of these were then placed on the short photoperiod bench and the remainder left on the long photoperiod bench, and used as controls.



FIG. 2.—A pair of plants approach-grafted. All leaves of plant on left subjected to short photoperiod during a part of experimental period. All parts of plant on right exposed to continuous long photoperiod.

Twenty more plants were approach-grafted as illustrated in figure 2. One leaf of one plant of each of five of the pairs was given short photoperiod. Thus with the exception of a single leaf, all the above-ground parts of both plants of each pair were subjected to long photoperiod. All the leaves of one plant of each of the remaining five grafted pairs were subjected to short photoperiod during the

first week of the treatment. As the plants grew and more foliage was produced than could be included in the box, the cover was removed from about the stem and the lid replaced at a higher level. Thus not all of the leaves were continuously exposed to short photoperiod. Such a grafted pair is shown in figure 2 just before being harvested. The boxes were put in place and removed at approximately the same hour as were the curtains on the short photoperiod bench.

In order to determine whether the artichoke can be photoperiodically induced, eighteen pots were transferred to the short photoperiod bench April 20. These were returned to the long photoperiod bench in groups of three after being exposed respectively to treatments of one, two, four, eight, twelve, and sixteen short photoperiods accompanied by correspondingly long dark periods.

### Results and discussion

On June 9 all the plants were removed from the pots, the soil carefully washed away from underground portions, and tuber formation noted. In order to show tubers and stolons more clearly, most of the roots were removed before the plants were photographed. The results are shown in table 1 and in figures 3 and 4.

Of the six plants which had only the terminal buds and young expanding leaves on short photoperiod (fig. 1), only one showed the slightest indication of tuber formation, and these tubers were few in number and very small (fig. 3B).

Additional evidence that the stem tip is not the locus of photoperiodic perception is furnished by the six plants whose stem tips were exposed to short photoperiod and the young leaves trimmed off repeatedly. None of these produced any tubers, although the six plants similarly trimmed and exposed to short photoperiods produced as numerous and as large tubers as did the controls on short photoperiod.

Eleven plants (including five of the grafted ones) had single leaves subjected to short photoperiod while all other above-ground portions were on long photoperiod. Without exception these produced tubers (figs. 3C, 4B), although they were not so large as were those on the short photoperiod controls and did not become evident until several days after thickening stolons were first noted on short photoperiod

control plants. This evidence leads to the conclusion that when exposed to short photoperiod, changes take place in the leaves of *Helianthus tuberosus* which lead to tuber formation.

TABLE 1  
TUBER FORMATION IN ARTICHOKES IN RESPONSE TO  
VARIOUS PHOTOPERIODIC TREATMENTS

TREATMENT	TOTAL NO. OF PLANTS	NO. OF PLANTS WITH TUBERS	NO. OF PLANTS WITHOUT TUBERS
LONG PHOTOPERIOD CONTROLS			
On continuous long photoperiod.....	6	0	6
Terminal bud and leaves up to 2 inches in length on short photoperiod (fig. 1).....	6	1	5
Terminal bud on short photoperiod; young leaves repeatedly trimmed.....	6	0	6
Short photoperiod with young leaves repeatedly trimmed from terminal bud.....	6	6	0
Long photoperiod with young leaves repeatedly trimmed from terminal bud.....	6	0	6
Long photoperiod with one leaf given short photoperiod.....	6	6	0
Long photoperiod grafted to plants whose upper portions were on short photoperiod (fig. 2).....	5	4	1
Long photoperiod grafted to plants which had only one leaf on short photoperiod.....	5	3	2
Long photoperiod except for induction period of:			
One short photoperiod and one long night.....	3	0	3
Two short photoperiods and two long nights.....	3	0	3
Four short photoperiods and four long nights....	3	0	3
Eight short photoperiods and eight long nights....	3	0	3
Sixteen short photoperiods and sixteen long nights.	3	3	0
SHORT PHOTOPERIOD CONTROLS			
Plants on continuous short photoperiod from April 18 to June 9.....	6	6	0

The grafted plants (fig. 4A, B) furnish evidence which, while not conclusive, tends to confirm that part of HITCHCOCK and ZIMMERMAN's suggestion that tuberization may be controlled by some substance with hormone-like properties. It was noted that there was a relationship between degree of development of the graft union and

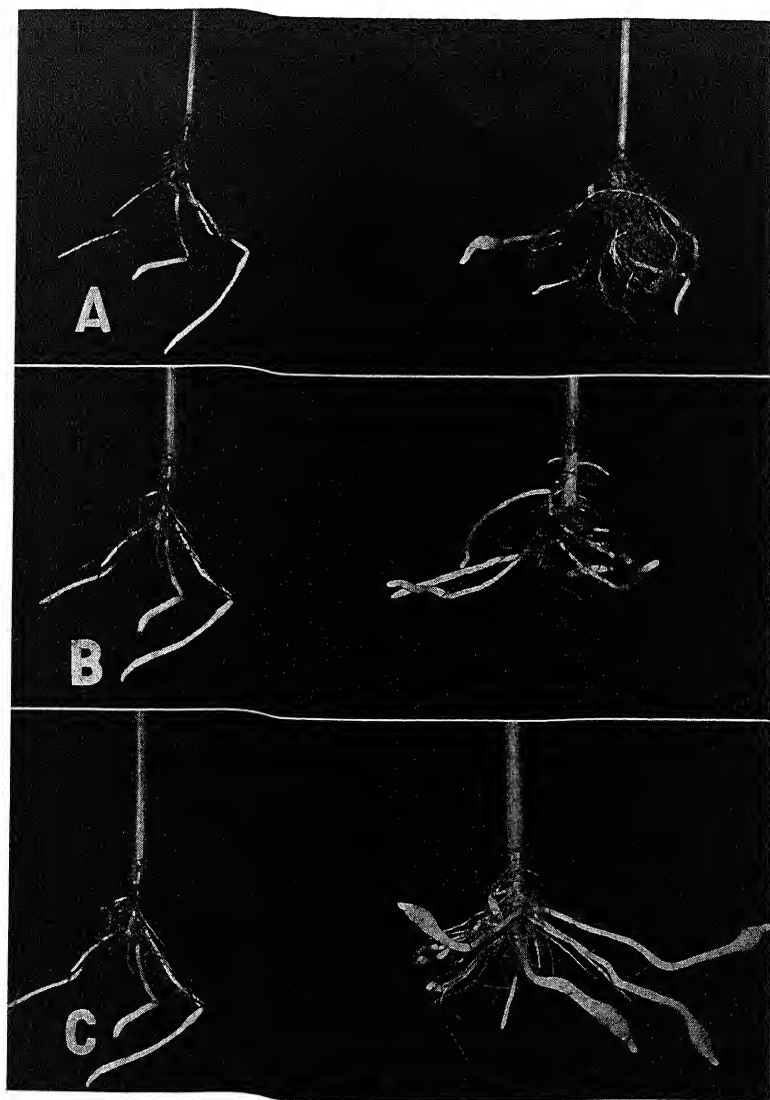


FIG. 3.—Plants at left in each case are the controls, grown on continuous long photoperiod. Plants on right received treatment as follows: *A*, grown on long photoperiod except for induction period of 16 short photoperiods beginning when plants were a little over two weeks old; *B*, terminal bud and leaves up to 2 inches in length maintained on short photoperiod; *C*, one leaf exposed to short photoperiod.

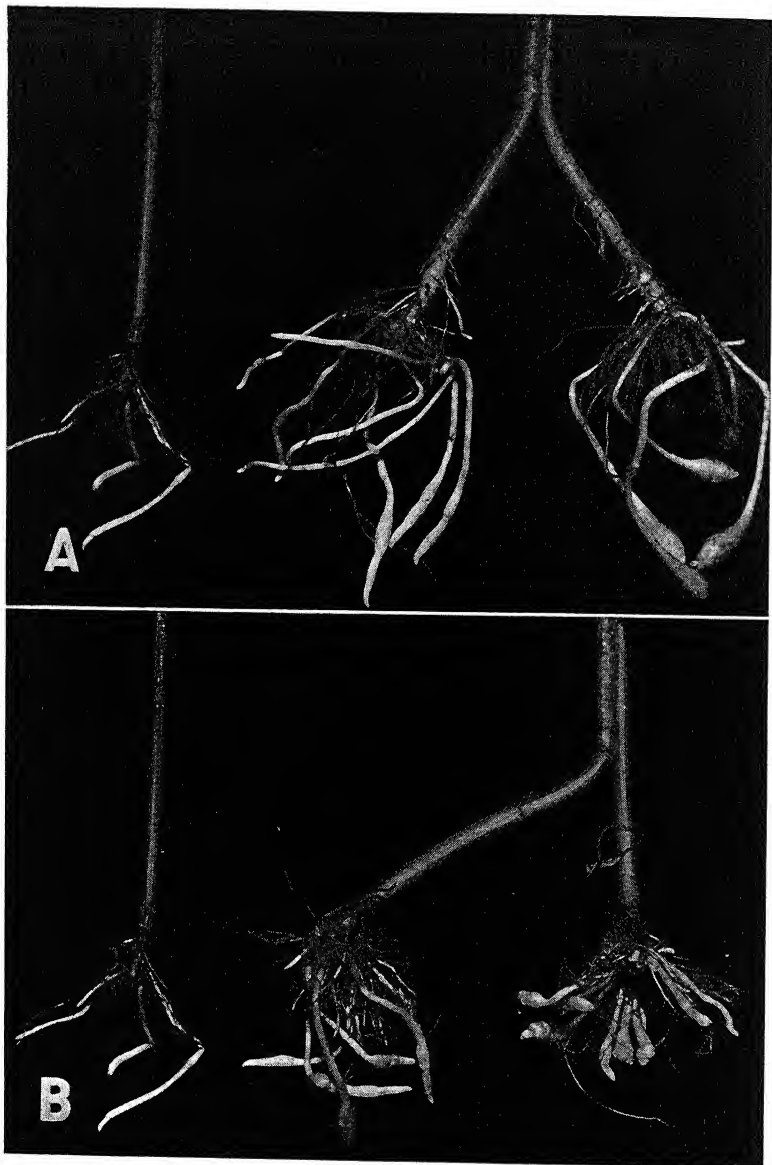


FIG. 4.—Plants at left in each case are the controls, grown on continuous long photoperiod. *A*, plant in center maintained continuously on long photoperiod but approach-grafted to plant on right, upper leaves of which were subjected to short photoperiod; *B*, plant in center maintained on long photoperiod but approach-grafted to plant on right, one leaf of which was subjected to short photoperiod.

size of the tubers of the receptor (4) plant. In those cases in which there was no evidence of transmission of a tuber-forming substance, the grafted stems fell apart when the raffia binding was removed. Whether there would have been transmission through a diffusion contact, as is true of a floral inducing substance in *Xanthium* (4), is not known.

While the tubers produced by the three plants subjected to an induction period of but sixteen short photoperiods were neither very large nor very numerous (fig. 34), it seems probable that artichokes are photoperiodically induced in relation to tuber formation.

ZIMMERMAN and HITCHCOCK do not state that they included leaves in the black bags placed over the terminal buds of their plants, but they mention no measures taken to avoid that situation, nor do they indicate the dimensions of the bags. Their results can be reconciled with those presented here only by assuming that they did include leaves somewhat more mature than those exposed to short photoperiods in the small boxes in these experiments. If fairly large leaves were included in their treatment of the tip of the plant, then it may be that these leaves induced tuber formation while they were being exposed to the short photoperiod, or that they were photoperiodically induced and stimulated tuber formation even after they became too large to be included in the bags.

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# A PLANT GROWTH INHIBITOR AND PLANT GROWTH INHIBITION

WILLIAM S. STEWART

(WITH ELEVEN FIGURES)

## Introduction

When auxin is applied unilaterally to the top of an *Avena* coleoptile there results a negative growth curvature; that is, a curvature away from the side on which the auxin is placed. During the course of investigations of an ether extract of radish cotyledons, it was ob-

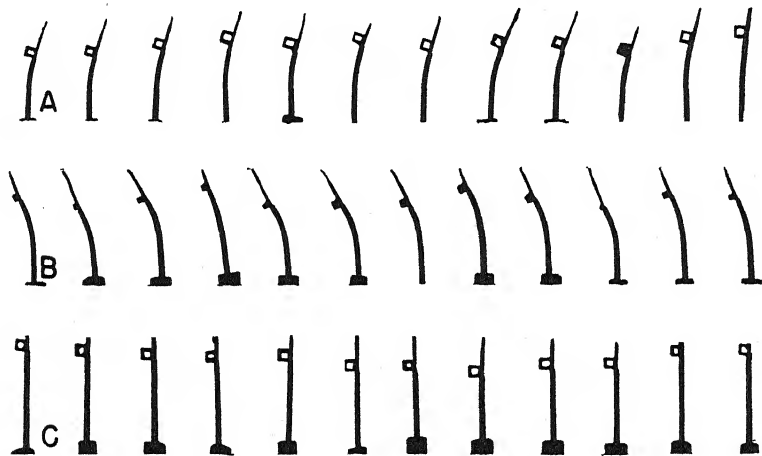


FIG. 1.—Growth curvatures of *Avena* coleoptiles: A, negative curvature, growth promotion by indoleacetic acid; B, positive curvature, growth inhibition by radish cotyledon extract; C, control, no curvature, blank 1.5 % agar.

served that the curvature was toward the side of application; that is, the curvature was positive (10). Examples of both positive and negative curvatures resulting from the application of growth substances are given in figure 1.

VIEHMAN (11) has found that lanolin pastes of acetic and formic acid in concentrations of from 0.0625 to 0.25 M, when applied to the top of a piece of *Helianthus* or *Lupinus* hypocotyl 1 cm. long, cause a substance to diffuse out of the bottom of the pieces which is capable

of eliciting positive *Avena* curvatures. These curvatures appear after two hours, and hence are not the same as those resulting from the inhibitor from radish which appear in less than one hour (fig. 4).

STARK (9), NIELSEN (3), and SEUBERT (7) as early as 1921 reported positive curvatures, but these were probably not caused by growth inhibiting substances as these investigators stated, but by the unequal production of auxin on the two sides of the top of the decapitated *Avena* coleoptile (14). Blank agar blocks applied to the top of such coleoptiles will cause this condition and hence positive curvatures after two and a half hours (fig. 5). GORTER (1), who first made this observation, states: "A growth retarding substance has not been found. Its appearance should cause a positive curvature of the stump within two and a half hours."

Since heretofore no substance had been found which so completely met the criteria for a true growth inhibitor, it became the object of this work to investigate the properties of this extract. In subsequent discussion reference made to growth inhibitor will refer to this substance obtained by ether extraction of radish cotyledons, variety French Breakfast, according to the method of VAN OVERBEEK (5). Inhibitor was applied to the test plants in 1.5 per cent agar blocks  $2 \times 2 \times 2$  mm. in size. The Siegeshafer strain of *Avena* was used according to the technique outlined by WENT and THIMANN (14).

### Investigation

#### BIO-ASSAY

The positive curvatures resulting from the action of the inhibitor suggested that they might be used as a quantitative biological assay for the inhibitor in the same manner that the negative *Avena* curvatures are used for the quantitative determination of growth promoting substances (14). To make certain of this, three factors had to be considered: (1) rate of curvature of the *Avena* coleoptile when treated with the inhibitor; (2) relation between degrees of positive curvature and concentration of the inhibitor; and (3) type of *Avena* test plant to use.

The rate of curvature was determined by the use of a photokymograph (6). In this device the movement of the *Avena* coleoptile is

recorded photographically every four minutes over a period of five to six hours following application of the substance being tested.

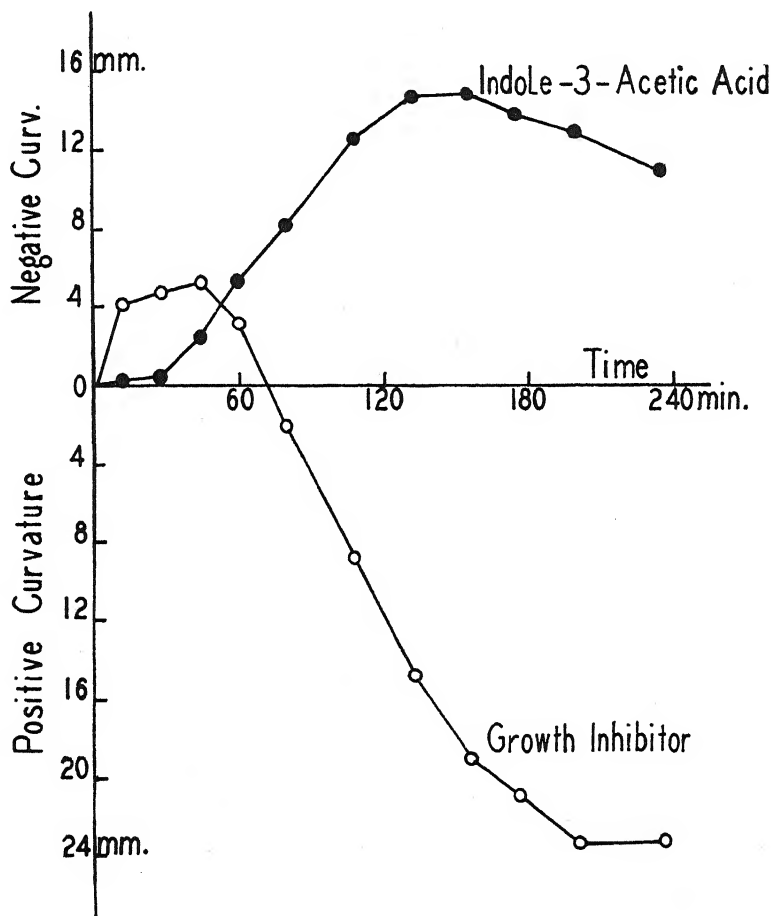


FIG. 2.—Curvature rate of once decapitated *Avena* plants upon application of: A, 0.05 mg./l. indoleacetic acid; B, growth inhibitor. Ordinate values given as mm. deviation of extended coleoptile from vertical position. Each point averages 12 test plants.

When this technique was used with the inhibitor extract it was found that after 150 minutes the maximum rate of positive curvature was still in progress, but soon thereafter decreased. The time for measuring the positive curvature was set at 150 minutes after application

of the substance being tested, instead of after 90 minutes as in the standard *Avena* test (fig. 2).

To investigate the relation between degrees of positive curvature and concentration of the inhibitor, a dilution series of inhibitor extract was prepared. Each dilution value was tested with 48 *Avena* plants. It was found that between  $3^\circ$  and  $13^\circ$  positive curvature there exists a linear proportionality to the concentration of the inhibitor (fig. 3).

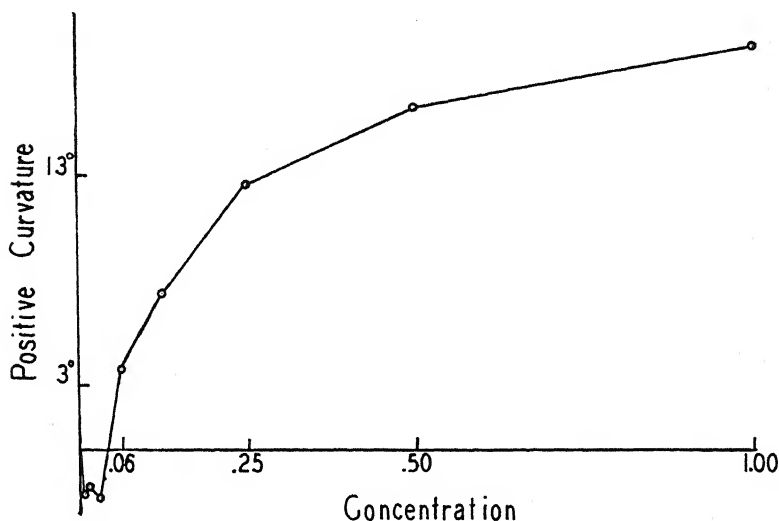


FIG. 3.—Relation between positive curvature of *Avena* plants and twofold dilutions of inhibitor substance.

By testing on the kymograph “deseeded” (8) once and twice decapitated *Avena* coleoptiles, and coleoptiles to which auxin had been added two hours before the test by applying it at the tip of the coleoptile in a small drop of lanolin (concentration 1:10,000), it was found that once decapitated plants showed the greatest response to inhibitor (fig. 4).

From these experiments the standard inhibitor test is defined as being the same as the standard *Avena* auxin test (14), except that: (1) the *Avena* coleoptile growth curvatures are positive; (2) the curvatures are measured after 150 minutes; and (3) the curvature is linearly proportional to the concentration of the inhibitor only be-

tween 3° and 13°. Although plants once decapitated show the greatest response to inhibitor, the difference between them and those

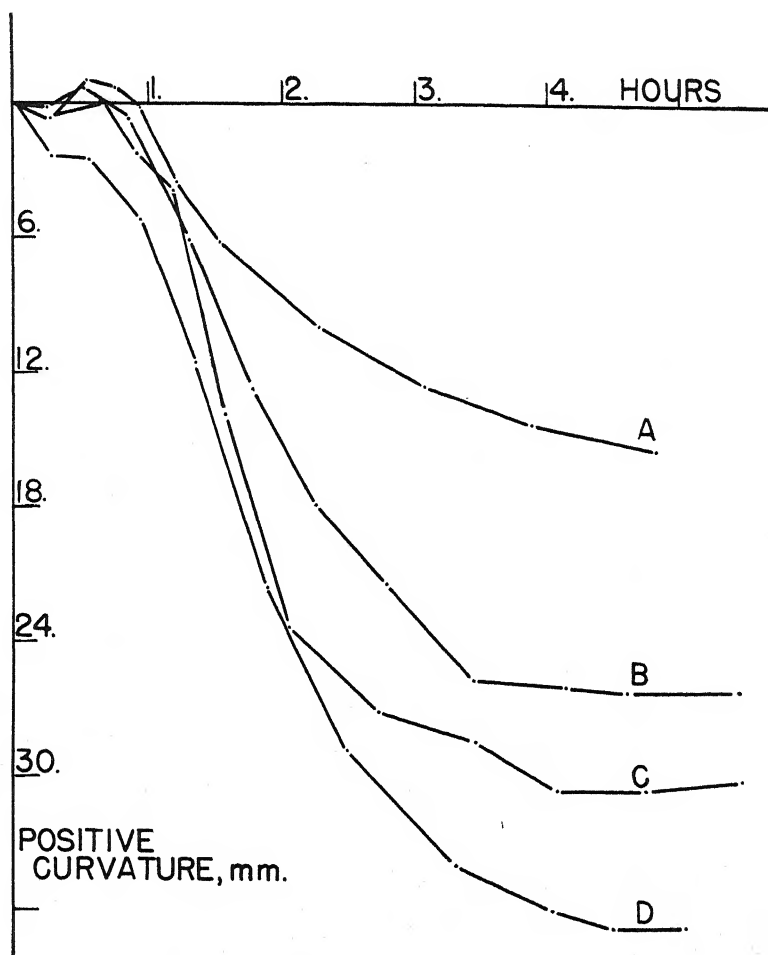


FIG. 4.—Inhibitor reaction rate of different types of *Avena* test plants: A, deseeded; B, added indoleacetic acid; C, twice decapitated; D, once decapitated. Each point averages 12 plants.

twice decapitated, after 150 minutes, is so slight that the twice decapitated coleoptiles were used as a matter of convenience except in special experiments.

## SOURCES OF INHIBITOR

Using this standard method for the inhibitor test, possible sources of inhibitor were sought. It was found in the cotyledons of six varieties of radish, in the cotyledons of turnip, rutabagas, mustard, and

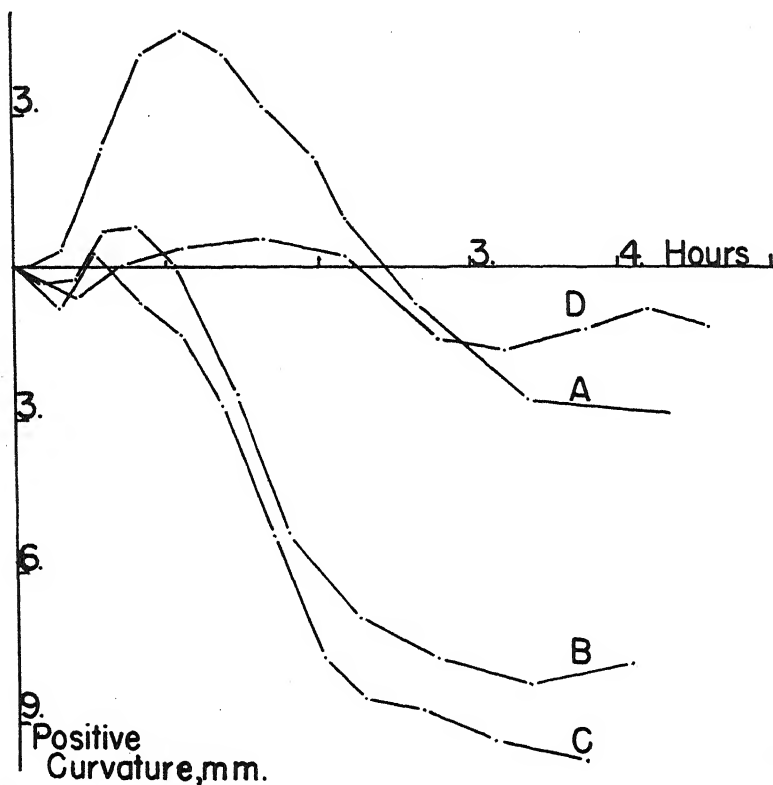


FIG. 5.—Reaction rate of twice decapitated *Avena* coleoptiles to purified inhibitor mixed with indoleacetic acid, 0.045 mg./l. A, inhibitor in ratio of 1:4 of indoleacetic acid; B, inhibitor in ratio of 4:1 of indoleacetic acid; C, inhibitor alone; D, blank agar. Each point averages 10–12 coleoptiles.

nasturtium; and it has been reported in the fruit of the pepper tree by A. J. HAAGEN-SMIT (unpublished work).

If both auxin and inhibitor were to occur simultaneously in an extract of the same material, the net curvature might be neither a measure of auxin nor of inhibitor. Only if the inhibitor were in high

enough concentration to cause a greater positive curvature than the negative curvature caused by auxin might its presence become apparent. Likewise only if the auxin were in sufficient concentration to cause a greater negative curvature than the positive curvature caused by the inhibitor would auxin become apparent. These two sets of conditions were realized by mixing indoleacetic acid (45 gamma per liter) with purified inhibitor in the ratios of 1:4 and 4:1. Figure 5 shows that the 4:1 ratio caused a  $4^\circ$  negative curvature, while after the same time (90 minutes) the 1:4 ratio caused a  $3^\circ$  positive curvature. Inhibitor with no indoleacetic acid caused a  $4^\circ$  positive curvature. (On the ordinate, 1 mm. deflection of the coleoptile equals approximately  $1^\circ$  curvature.) Thus in an ether extract of plant tissue depending on the relative amounts of auxin and inhibitor present, the *Avena* test would give curvatures that would be true measures neither of auxin nor of inhibitor.

Indirect evidence for the presence of inhibiting substances in ether extracts that mask the effect of auxin has recently been presented by GOODWIN (2).

From these considerations it seems likely that the occurrence of inhibitor may be rather widespread, but that it is overlooked because it occurs in too low a concentration and is accordingly masked by the auxin.

#### INHIBITOR IN GROWING RADISH PLANT

Radish seeds, French Breakfast, were sown in flats containing loamy soil, at intervals of three days over a period of two and a half months. During the last two weeks of this period seeds were planted every day. At the end of the two and a half months the amounts of inhibitor in the cotyledons and leaves of plants of the various ages were determined. Ether extractions of the hypocotyls showed no curvatures. Additional results are given in figure 6. A few determinations on plants of various ages were repeated at another time and found to agree with those presented here. As the plant grows the amount of inhibitor reaches a sharp maximum on the eighth day after the seeds are planted, and from then on decreases until the cotyledons finally contain no inhibitor but do contain auxin. The developing leaves, from the youngest at the tip to the fully expanded

ones, show a similar continual decrease in the amount of inhibitor with aging, but there is no indication at any time of an increase in inhibitor content as there is in the cotyledons. While figure 6 shows that the concentration of inhibitor per gram fresh weight of the leaves decreases with growth, it does not necessarily follow, because of change in size or weight of the leaf, that the amount of inhibitor per leaf decreases accordingly. A comparison of the amount of in-

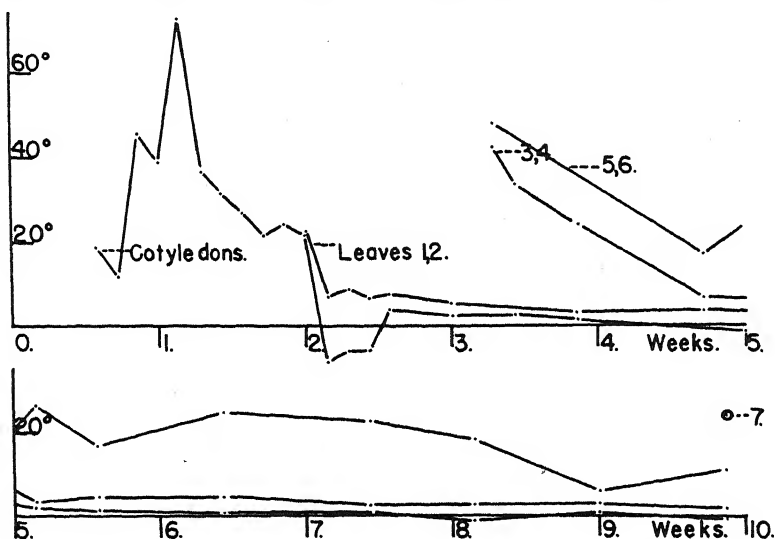


FIG. 6.—Inhibitor distribution with age in cotyledons and leaves of radish. Leaves numbered in order of appearance. Each point averages 12 test plants. Inhibitor given as degrees positive curvature per 5 gm. fresh weight material when extract is taken up in 1 cc. of 1.5 % agar.

hibitor per leaf shows that the two oldest leaves of plants 52 days old had enough inhibitor per leaf, calculated on the basis of a 5-gm. fresh weight sample extracted and taken up in 1 cc. of 1.5 per cent agar, to cause 3.5° positive curvature, while comparable leaves 69 days old showed a 2.5° negative curvature. Each of the next two oldest leaves had sufficient inhibitor to cause 25° positive curvature, while comparable leaves 69 days old showed 5° positive curvature per leaf. It is thus apparent that with growth the total amount of inhibitor per leaf does actually decrease until it is no longer detectable and only auxin curvatures result from the ether extracts.

## CHEMICAL PROPERTIES OF INHIBITOR SUBSTANCE

Mr. C. E. REDEMANN, working with Dr. A. J. HAAGEN-SMIT, began an investigation (unpublished) of the chemical properties of the inhibitor substance. It was found that when an active inhibitor extract was tested for its stability in acid or base, the positive curvature activity was completely lost and replaced by a strong negative curvature activity upon the alkaline hydrolysis. This made it seem likely that the substance causing the positive curvatures was a precursor of one of the auxins. It was also found that the inhibitor contained no acidic or basic groups, making it necessary to postulate for the inhibitor a compound, part of which was this auxin, but without its free carboxyl group. From the pronounced solubility in fat of the inhibitor, it seemed possible that an ester linkage between the auxin part of the molecule and the remaining portion might exist. Further work on this point is necessary.

The well established chemical properties of the inhibitor are: (1) it can be extracted from plant tissue with either di-ethyl ether or ethyl acetate; (2) it contains no acidic or basic groups; (3) it can be easily hydrolyzed to yield a growth promoting fraction; (4) certain chemical tests indicate that the growth promoting fraction is indole-acetic acid.

EFFECT OF INHIBITOR ON GROWTH OF *AVENA* COLEOPTILES

A growth rate which is relatively less on the treated side causes a positive curvature. This relative difference in growth rate could be brought about either by an actual inhibition of growth on the treated side or by an increase of growth on the untreated side. Growth rate measurements were made as follows. The upper 15 mm. of intact *Avena* coleoptiles were marked into 3 mm. zones with India ink. Growth of these zones was then followed at 2-hour intervals by measuring the increase in distance between them. To one set of six plants purified inhibitor was applied over the tip millimeter of the coleoptile in a small drop of lanolin paste. To another set of six plants pure lanolin paste alone was applied. Throughout the course of the experiment the plants were kept in the regular *Avena* testing dark-rooms, where the humidity and temperature were maintained con-

stant at 85 per cent and 24° C. The plants were illuminated only with light transmitted through a Corning orange light filter, no. 243. The growth of these two sets of plants was measured accurately with a horizontal microscope to 0.01 mm.

The inhibitor decreased the growth rate to about one-fourth that of the controls, except in the lowest zone where it was about one-half that of the controls (fig. 7). The inhibitor, at the concentration used, stopped growth of the coleoptiles in six hours, whereas the con-

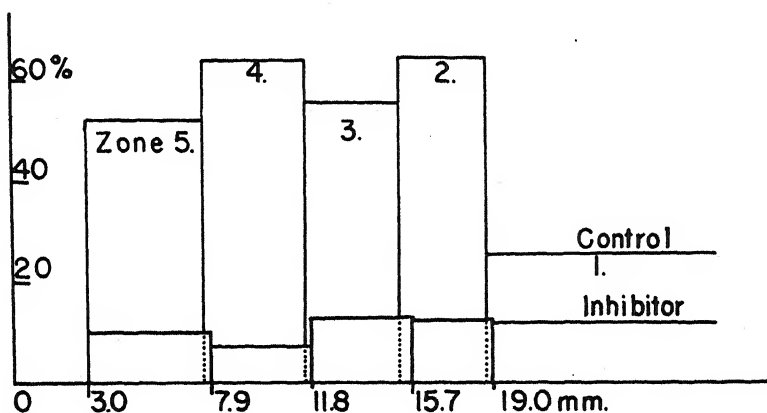


FIG. 7.—*Avena* coleoptile growth distribution when treated with inhibitor. Ordinate, percentage increase in length during 12 hours. Abscissa, coleoptile zones in mm. from tip.

trols continued to grow for six hours longer. The growth inhibition took place equally in all zones except the lowest one, where there was stimulation followed by inhibition (fig. 8). Thus it may be concluded that the relative decreased growth rate of the *Avena* coleoptile on the side treated with inhibitor is caused by an actual inhibition of growth on that side, rather than by an increase in the growth rate on the untreated side.

These results were substantiated by growth measurements on isolated sections of *Avena* coleoptiles. In these experiments 4.2 mm. sections were cut from *Avena* coleoptiles or radish hypocotyls and placed in inhibitor solutions of different concentrations. In all concentrations except the very lowest, a growth inhibition took place

when compared with growth in water alone. The very lowest inhibitor concentration showed the same amount of growth as the water controls.

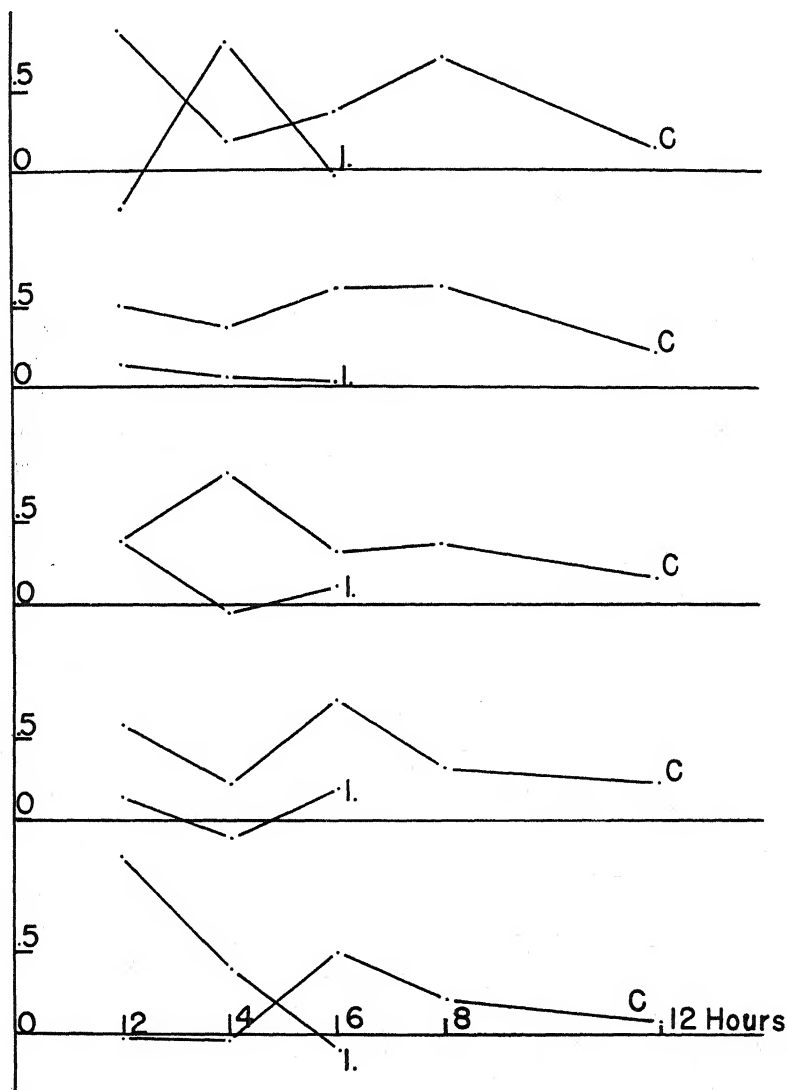


FIG. 8.—Distribution of growth rate of *Avena* coleoptiles when treated with inhibitor. Ordinate, increase in zone length in mm. C, control; I, inhibitor treated plants.

## TRANSPORT POLARITY OF INHIBITOR

It has been pointed out that the inhibitor is a substance of neutral chemical character; that is, the molecule contains neither acidic nor basic groups. Accordingly it could be expected, on the basis of WENT's (13) potential gradient theory of auxin transport, that inhibitor should be transported acropetally as well as basipetally. Experiments have shown this to be the case, as equal amounts of inhibitor were found to pass through uninverted as well as inverted sections of *Avena* coleoptiles.

Similar data were found for sections of radish hypocotyls, except in one experiment where, instead of finding inhibitor in the agar blocks at the lower end of the sections being used, auxin was found. Attempts to repeat this experiment were unsuccessful and showed only inhibitor passing through the uninverted and inverted sections. Inhibitor can mask the effect of auxin, and since in this one instance auxin was found to be present in the lower blocks of the sections of radish hypocotyl which transported the inhibitor, but in no other cases, a method was devised whereby a time record could be made of any growth promoting or growth inhibiting substances which diffused from the sections. Thus subsequent masking of the first substances diffusing out of the sections was overcome. This method consisted of mounting a 4.2 mm. long section of radish hypocotyl on the shoulder of a decapitated *Avena* coleoptile. On top of the radish section was placed an agar block containing the inhibitor. The curvatures of the coleoptile were recorded photographically by means of the photokymograph.

## REMOVAL OF AUXIN FROM CRUDE INHIBITOR EXTRACT

Before investigating the substances (possibly auxin) being produced by the transport of inhibitor through plant tissues, it was first necessary to be certain that there was no auxin contaminating the original inhibitor preparation.

Since it was impossible to purify the inhibitor by any known chemical means without the risk of hydrolyzing it into auxin, a physiological purification method was developed. It is well established that *Avena* coleoptiles will transport auxin only basipetally. Inhibitor substance on the other hand can be transported in both directions.

Consequently the technique was to pass inhibitor through inverted *Avena* coleoptile sections 4.2 mm. long. Because they were inverted the auxin was prevented from passing through but the inhibitor could be collected as it diffused out of the section into an agar block

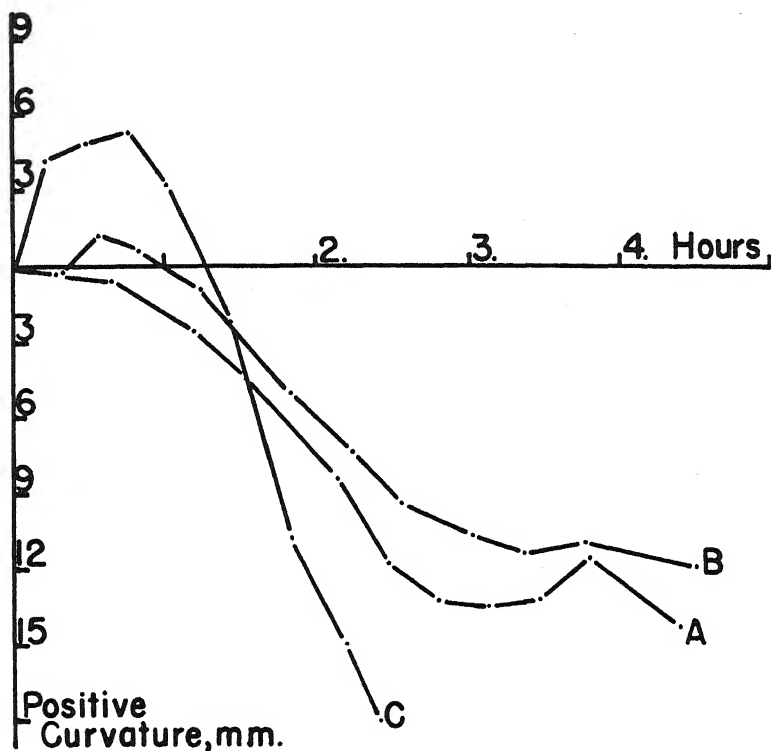


FIG. 9.—Reaction of twice decapitated *Avena* coleoptiles to purified inhibitor, two concentrations, 1 and 0.25 (A and B), and of once decapitated coleoptiles to unpurified inhibitor (C). Each point averages 9–12 coleoptiles.

at the other end. Generally ten to twelve hours' transport time was allowed for the preparation of such "purified" inhibitor (fig. 9). This experiment has been repeated many times with similar results.

The reaction to crude inhibitor extract; that is, a negative curvature followed by a positive one can be duplicated by using purified inhibitor and mixing indoleacetic acid with it. When this is done the same type of curvature is found as that obtained with the crude

inhibitor extract (figs. 5, 2). Apparently the negative curvatures preceding the positive curvatures (and resulting from application of the crude unpurified extract) are caused by auxin which was extracted from the cotyledons together with inhibitor.

#### HYDROLYSIS OF INHIBITOR AT CUT SURFACES

The one experiment which indicated that auxin diffused from inverted as well as from uninverted radish hypocotyl sections was re-

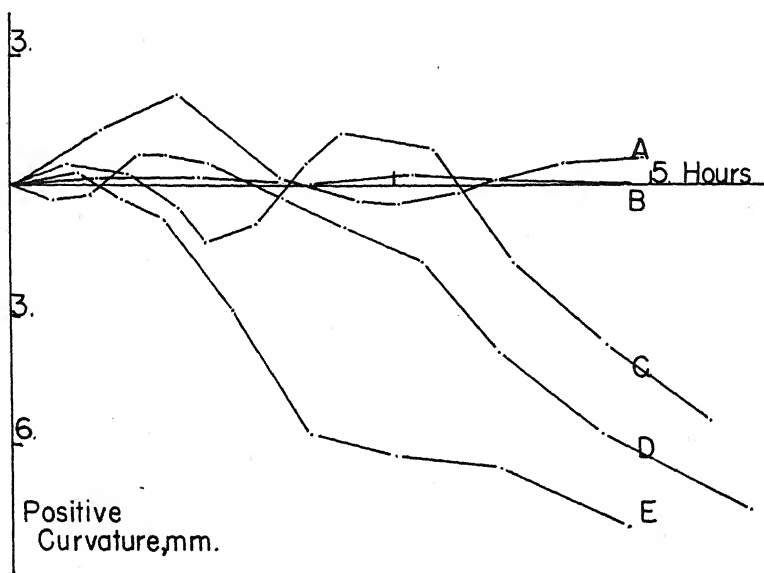


FIG. 10.—Reaction rate of twice decapitated *Avena* coleoptiles to purified inhibitor transported through inverted and uninverted 4.2 mm. sections of radish hypocotyl. Each point averages 12 coleoptiles. A, inverted section with blank agar; B, uninverted section with blank agar; C, inverted section with inhibitor; D, uninverted section with inhibitor; E, inhibitor applied directly, no section intervening.

peated by the kymograph technique, using auxin-free inhibitor. Negative curvatures were initiated even when the inhibitor was passed through the inverted sections which are known not to transport auxin. These initial negative curvatures soon were masked with the transport of more inhibitor and became positive (fig. 10). This experiment was repeated with sections of *Avena* coleoptiles with essentially the same results (fig. 11). Both experiments have been

repeated several times with similar results. From these experiments it appears that here, as well as in the one exceptional experiment, a small amount of inhibitor was hydrolyzed into auxin at the cut surfaces of the transporting sections and test plants.

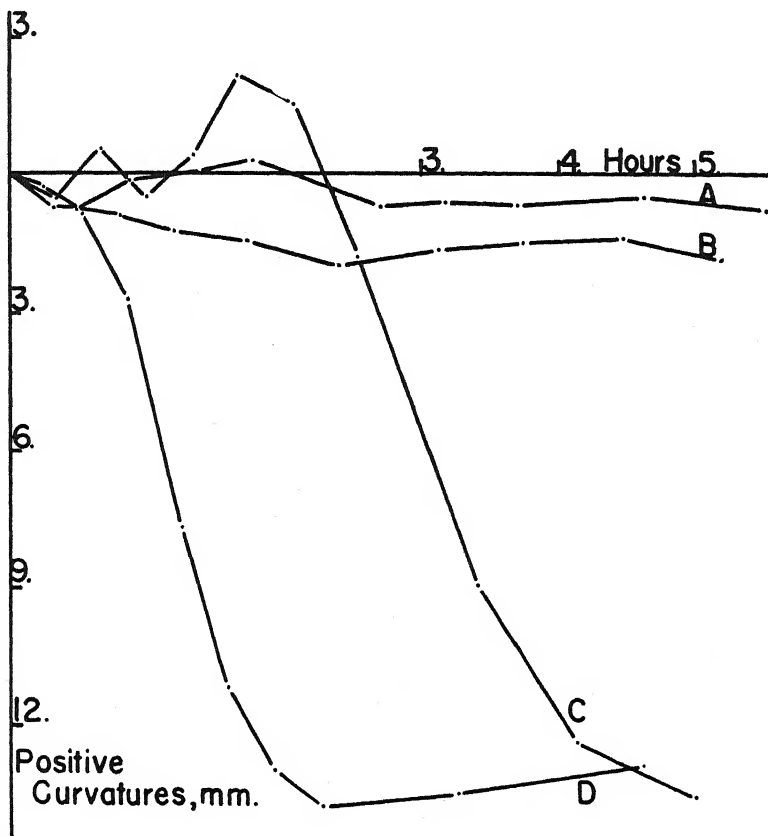


FIG. 11.—Reaction rate of twice decapitated *Avena* coleoptiles to purified inhibitor transported through inverted 4.2 mm. sections of *Avena* coleoptiles. A, B, average of 6 coleoptiles; C, D, average of 12 coleoptiles. A, blank agar applied directly, no section intervening; B, indoleacetic acid, 0.5 mg./l., inverted section; C, purified inhibitor, inverted section; D, purified inhibitor applied directly, no section intervening.

From this and similar experiments, the velocity of the transport of inhibitor either acropetally or basipetally through *Avena* coleoptile sections was found to be 11 mm. per hour, or the same as the transport velocity of auxin.

## PHYSIOLOGICAL ROLE OF INHIBITOR

In many different tests of physiological activity, such as the pea test for growth substances, root growth inhibition test, seed germination, etc., inhibitor was found to act like auxin because during the course of the test it actually was hydrolyzed into auxin. In the case of bud inhibition, where the inhibitor was used in anhydrous lanolin paste and accordingly could not hydrolyze easily, it was unable to act like auxin and did not inhibit the outgrowth of buds. This experiment, however, is inconclusive since the inhibitor paste caused injury, so that no active substances could reach the lower parts of the branch.

Whether inhibitor acts as a growth inhibitor in the radish plant itself is open to question.

It has been shown by VAN OVERBEEK (4) that an auxin diffuses from the cut surface of radish cotyledon petioles. No indication is given, however, as to whether this auxin is indoleacetic acid, auxin-a, or auxin-b. As one of the best means of distinguishing between the auxins is by their molecular weights, molecular weight determinations by the diffusion technique (12) were made for: (1) the auxin coming from the cut surface of radish cotyledon petioles; (2) the purified inhibitor; and (3) indoleacetic acid (control). The molecular weights of the auxin coming from the cotyledons and of the inhibitor itself were determined in three experiments. In the case of the auxin, twelve *Avena* test plants were used to determine the relative concentration in each of the agar plates. In the case of the inhibitor, thirty-six (or in one instance forty-eight) test plants were used. Values determined for the molecular weight of the auxin coming from the cotyledons were 144, 104, and 125. The values for the molecular weight of the inhibitor were 142, 117, and 83. The value for the control, indoleacetic acid, known molecular weight of 175, was 159.

The diffusion method of determining molecular weights is not so useful for establishing exact molecular weights as it is for indicating substances of known molecular weight. In this case the auxin diffusing from the cotyledons is apparently neither auxin-a nor auxin-b (molecular weights 328 and 310); it may be indoleacetic acid. A

chemical study of the auxin obtained by hydrolysis of inhibitor likewise indicates that it may be indoleacetic acid. It is interesting to speculate as to the likelihood of the diffusing auxin originating by hydrolysis of the inhibitor in the cotyledons.

### Summary

1. There is an ether-extractable substance in the cotyledons and leaves of radish plants that is capable of causing growth inhibition and positive *Avena* coleoptile curvatures.
2. It is possible to analyze for this substance on a quantitative basis.
3. This substance occurs in other plants as well as radish.
4. With growth of radish leaves their inhibitor content decreases and is finally replaced by auxin.
5. This inhibiting substance has no acidic or basic groups and can readily be hydrolyzed to form a growth promoting substance.
6. Inhibitor has no polarity of movement in the radish or *Avena* plant.
7. Auxin contaminating the crude inhibitor extract may be removed by use of the "inverse transport" purification method.
8. Inhibitor may be hydrolyzed at cut plant surfaces to form auxin.
9. Inhibitor is transported through *Avena* coleoptiles at approximately 11 mm. per hour, or at the same rate as auxin.
10. In many tests for physiological activity, inhibitor behaves like auxin.
11. The auxin diffusing from radish cotyledons is possibly indoleacetic acid.

This work was done at the California Institute of Technology, Pasadena, California.

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# EFFECTS OF SOIL TEMPERATURE, pH, AND NITROGEN NUTRITION ON THE DEVELOPMENT OF *POA PRATENSIS*<sup>1</sup>

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 503

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## Introduction

Few studies have been made in which plants were grown under the combined influence of controlled nitrogen nutrition, temperature and reaction of the culture medium. In the present series of experiments bluegrass, *Poa pratensis* L., was chosen as a representative plant, sometimes grown in the field under semi-controlled nutrient conditions and differing widely in development with seasonal variation in temperature and soil reaction. The experimental plants were grown with a controlled supply of nitrogen as nitrate or ammonium, under controlled soil temperatures and reaction of the culture media. Measurements of leaf and root growth were taken to express development under the environmental conditions maintained and as a measure of the effects of periodic clipping.

The influence of the reaction of the culture medium on absorption and assimilation of nitrate and ammonium ions has been studied by many workers. NIGHTINGALE (11) has critically reviewed the literature dealing with nitrogen nutrition of green plants, and PARDO (14) reviewed the status of ammonium nutrition. The relation of temperature to nitrogen nutrition has been studied by HARRISON (5) on *Poa pratensis* and by NIGHTINGALE (9, 10, 12, 13) on several plants, including tomatoes, apple trees, and peach trees. Numerous field studies have been conducted on bluegrass, and several greenhouse experiments, notably those by HARRISON (4, 5), AHLGREN (1), GRABER (2), and GRABER and REAM (3), have been concerned with clipping effects.

<sup>1</sup> Supported by a Research Fellowship of the United States Golf Association Green Section.

## Experimental data and results

### UNCLIPPED SERIES

ELEVEN-WEEKS EXPERIMENT.—To determine the effects of nitrate and ammonium nutrition at varying pH and soil temperatures on the development of unclipped *Poa pratensis* plants, an experiment was set up using the temperature tank apparatus described by LINK (6).

Plants obtained by vegetative propagation from a single plant were grown in 2-gallon crocks filled with acid-washed quartz sand. Two nutrient solutions, one containing nitrogen in the form of nitrate and the second in the form of ammonium, were made up in the usual manner, according to the concentrations outlined in table 1.

TABLE 1  
COMPOSITION OF NUTRIENT SOLUTIONS (PARTIAL VOLUME MOLECULAR  
CONCENTRATIONS OF SALTS USED)

SOLUTION	$\text{KH}_2\text{PO}_4$	$\text{Ca}(\text{NO}_3)_2$	$\text{MgSO}_4$	$\text{CaCl}_2$	$(\text{NH}_4)_2\text{SO}_4$
Nitrate.....	0.00211	0.00286	0.00710	.....	.....
Ammonium...	0.00211	.....	0.00710	0.00286	0.00286

These concentrations are taken from those of NAFTEL (8). Solutions of three pH values, 4.5, 5.5, and 6.5, were made from each of these stock solutions by the addition of N/10 NaOH or N/10  $\text{H}_2\text{SO}_4$  until the desired pH was obtained, colorimetrically measured. The experiment was run in quadruplicate, each of the six nutrient conditions being maintained at three soil temperatures, 15°, 25°, and 35° C. The plants were grown from March 3 to May 10, 1936, under greenhouse conditions and with a relatively large amount of sunshine.

Nutrient solution was added to each culture in 400 cc. amounts twice daily. Regulation of the nutrient medium to the desired pH was accomplished by flushing each culture with distilled water before each nutrient addition. Sufficient water at the desired pH was added to bring the cultures to the proper reaction as determined by testing the leachate colorimetrically. In this way the reaction of each culture was regulated twice daily and the change in pH of the nutrient

solution as indicated in table 2 was manifested over a period of ten hours. It will be seen from the table that plants supplied with nitrate nitrogen at three pH values did not appreciably change the

TABLE 2

WEEKLY pH READINGS ON CULTURE SOLUTIONS CONTAINING PLANTS  
GROWN FOR 11 WEEKS IN SAND AT THREE SOIL TEMPERATURES  
AND NITRATE AND AMMONIUM NUTRITION AT VARIED pH

No. OF WEEKS	15° C.			25° C.			35° C.		
	4.5	5.5	6.5	4.5	5.5	6.5	4.5	5.5	6.5
NITRATE									
1.....	5.0	5.4	6.4	5.0	5.5	6.5	5.0	5.7	6.5
2.....	5.0	5.6	6.4	5.1	5.7	6.4	5.1	5.8	6.4
3.....	5.1	5.7	6.6	5.1	5.6	6.2	5.2	5.8	6.5
4.....	5.0	5.8	6.6	5.0	5.6	6.2	5.1	5.6	6.4
5.....	5.1	5.7	6.5	5.0	5.4	6.0	5.1	5.5	6.2
6.....	5.0	5.5	6.6	4.0	5.3	6.5	5.0	5.6	5.4
7.....	5.0	5.5	6.5	5.0	5.4	6.3	5.0	5.6	6.2
8.....	4.8	5.6	6.4	4.6	5.4	6.5	5.0	5.6	6.3
9.....	5.0	5.5	5.8	5.0	5.0	6.2	5.0	5.6	6.2
10.....	5.8	5.4	6.2	5.0	5.6	6.3	5.0	5.2	6.3
AMMONIUM									
1.....	5.0	5.4	6.4	5.4	5.5	6.5	5.2	5.5	6.5
2.....	5.0	5.6	6.4	5.2	5.8	6.6	5.3	5.8	6.5
3.....	5.4	5.6	6.5	5.3	5.5	6.1	5.3	5.6	6.0
4.....	4.8	5.6	6.6	5.3	5.3	5.2	5.2	5.5	5.2
5.....	5.1	5.5	6.5	5.1	5.3	5.0	5.0	5.2	4.6
6.....	5.0	5.5	6.5	4.8	5.1	5.6	5.0	5.3	5.0
7.....	4.8	5.5	6.3	4.6	5.4	5.0	5.0	5.3	4.9
8.....	5.0	5.5	5.8	4.6	5.0	5.3	4.6	5.2	5.0
9.....	5.0	5.5	5.4	4.7	5.2	5.3	4.6	5.4	5.0
10.....	5.0	5.2	5.0	5.0	5.0	5.6	5.0	5.0	5.4

reaction of the culture medium over this half-day period, with the exception of the pH 4.5 solution. In general, both nitrate and ammonium solutions applied at pH 4.5 changed rapidly to pH 5.0 and remained fairly constant at that value. Plants supplied with ammonium at pH 6.5 brought about a decided increase in hydrogen ion concentration of the medium, especially at the higher temperatures.

Some increase was noted in the case of solutions supplied at a pH of 5.5.

Measurements were taken for each culture of the length and number of leaves, green and dry weights of roots and leaves, and the number of rhizomes produced. These data are given in tables 3 and 4. Table 3 shows the decided superiority of growth, as evi-

TABLE 3

LEAF AND RHIZOME DEVELOPMENT OF PLANTS GROWN FOR 11 WEEKS IN SAND AT THREE SOIL TEMPERATURES AND NITRATE AND AMMONIUM NUTRITION AT VARIED PH. AVERAGES BASED UPON FOUR PLANTS

TEMPERATURE AND PH	PERCENTAGE INCREASE LEAF LENGTH		PERCENTAGE INCREASE LEAF NUMBER		PERCENTAGE LINEAR INCREMENT		No. OF RHIZOMES	
	NO <sub>3</sub>	NH <sub>4</sub>	NO <sub>3</sub>	NH <sub>4</sub>	NO <sub>3</sub>	NH <sub>4</sub>	NO <sub>3</sub>	NH <sub>4</sub>
15° C.								
4.5.....	198	31	955	85	3048	145	16.5	3.2
5.5.....	194	54	942	229	2514	408	18.0	2.8
6.5.....	191	144	1046	524	2847	1434	32.0	7.5
25° C.								
4.5.....	173	75	600	35	1842	265	28.0	1.0
5.5.....	233	115	401	148	1585	442	33.0	3.3
6.5.....	182	192	620	314	1799	1015	38.0	10.7
35° C.								
4.5.....	140	23	263	0	761	4	28.5	0
5.5.....	133	55	226	53	801	141	24.3	1.3
6.5.....	148	66	170	80	589	209	14.5	2.0

denced by increase in leaf length and number of leaves and rhizomes produced, of the nitrate-supplied plants as compared with ammonium-supplied plants under corresponding temperature and pH conditions. The data for linear increment (a combined measure of leaf length and number) show that nitrate-supplied plants produced almost twice as much growth as plants with ammonium nutrition under pH conditions most favorable for ammonium nutrition. Differences in reaction had no appreciable effect on leaf development of nitrate plants at 15° and 25° C. At 35° C., however, a slight increase in the number of leaves and rhizomes of pH 4.5 plants over the other plants at that temperature was noted. Ammonium plants showed

the least increase in length and number of leaves at pH 4.5 and the best development at pH 6.5 under all temperature conditions.

Temperature effects on leaf development were also evident. In all cases the least development occurred under the highest temperature conditions. The greatest number of leaves was produced at 15° C., whereas increase in leaf length was almost equal at 15° and

TABLE 4

GREEN AND DRY WEIGHTS OF PLANTS GROWN FOR 11 WEEKS IN SAND AT THREE SOIL TEMPERATURES AND NITRATE AND AMMONIUM NUTRITION SUPPLIED AT VARIED PH. AVERAGE WEIGHT (IN GRAMS) BASED UPON FOUR PLANTS

TEMPERATURE AND PH	LEAVES				ROOTS			
	GREEN WEIGHT		DRY WEIGHT		GREEN WEIGHT		DRY WEIGHT	
	NO <sub>3</sub>	NH <sub>4</sub>	NO <sub>3</sub>	NH <sub>4</sub>	NO <sub>3</sub>	NH <sub>4</sub>	NO <sub>3</sub>	NH <sub>4</sub>
15° C.								
4.5.....	17.0	1.1	3.40	0.26	12.6	2.4	4.06	0.85
5.5.....	20.5	2.1	3.48	0.58	14.4	3.1	3.96	1.07
6.5.....	20.1	8.1	3.59	1.71	17.0	7.5	4.50	2.65
25° C.								
4.5.....	10.7	1.0	2.58	0.24	14.0	2.3	5.62	0.91
5.5.....	13.8	2.7	3.27	0.88	12.8	3.2	4.25	1.10
6.5.....	13.1	7.5	3.13	1.77	13.5	7.5	4.78	2.00
35° C.								
4.5.....	5.0	0.8	1.62	0.14	7.0	1.6	3.31	0.44
5.5.....	5.0	1.2	1.56	0.27	7.0	1.7	2.91	0.65
6.5.....	3.5	1.9	1.13	0.43	5.0	2.5	2.21	0.87

25° C. If linear increment is used as a growth index, it is seen that greatest growth, in nitrate plants especially, occurred at the lowest temperature, with decided reductions at higher temperatures. No decided tendencies were noted in rhizome production with respect to temperature, although the maximum number in both nitrate and ammonium plants was produced at the median temperature.

Table 4 shows another index of growth under the conditions of the experiment in the form of green and dry weights of the leaves and roots. The decided superiority in growth of nitrate-supplied over ammonium-supplied plants was evident in all cases. Differ-

ences in weight of nitrate plants owing to pH effects were found only at the highest temperature, at which pH 4.5 plants were superior. Ammonium plants produced the greatest amounts of leaves and roots at pH 6.5. With both nitrate and ammonium nutrition, the root and leaf weights of plants grown at 15° and 25° C. were decidedly superior to corresponding plants grown at 35° C.

Examination of the root systems of the nitrate and ammonium plants showed a general similarity when grown at 15° and 25° C. High temperature caused the production of a root system only half the size produced at lower temperatures.

THREE AND SIX WEEKS EXPERIMENTS.—Two additional series were grown in order to furnish information concerning the early development of plants grown under the conditions of the previous experiment. Two plants were grown in each condition of nutrient supply, temperature, and pH. The procedure described in the previous experiment was followed with the exception that duplicate plants were placed in the same container. For the length of time the experiment was conducted, the amount of nutrient added was sufficient for both plants. The two series were planted on April 26, 1936, and nutrient treatments started on May 4 and continued to May 26 and June 12 respectively for the three and six weeks series. The weather conditions during this period were perhaps a little more favorable for plant growth than during the period of the previous eleven weeks experiment, as longer days and clearer weather prevailed.

Determinations of the reaction of the culture solutions were made at weekly intervals and showed essentially the same general trend in pH that prevailed in the previous experiment. At the end of the sixth week of growth, plants supplied with ammonium at pH 6.5 were active enough to convert the culture solution to pH 5.0. Some increase in acidity of the pH 6.5 solution containing nitrate was noted, but in general the nitrate-supplied plants effected little change in the acidity of the culture solutions.

The development of the plants in these two series was followed in the same manner as in the other experiment, and the results for leaf and rhizome production are shown in tables 5 and 6. The type of response to temperature and nutrient supply was similar in these

shorter series to that shown in the eleven weeks experiment. At the end of three weeks, plants supplied with nitrate at pH 6.5 had superior leaf development except under high temperature conditions, but observations at the end of six weeks showed little difference in pH effect. Ammonium plants showed the best leaf and rhizome development at pH 6.5 at all temperatures, and in all cases the de-

TABLE 5

LEAF AND RHIZOME DEVELOPMENT OF PLANTS GROWN FOR THREE WEEKS IN SAND AT THREE SOIL TEMPERATURES AND NITRATE AND AMMONIUM NUTRITION SUPPLIED AT VARIED PH. AVERAGES BASED UPON TWO PLANTS

TEMPERATURE AND pH	PERCENTAGE INCREASE LEAF LENGTH		PERCENTAGE INCREASE LEAF NUMBER		PERCENTAGE LINEAR INCREMENT		No. OF RHIZOMES	
	NO <sub>3</sub>	NH <sub>4</sub>	NO <sub>3</sub>	NH <sub>4</sub>	NO <sub>3</sub>	NH <sub>4</sub>	NO <sub>3</sub>	NH <sub>4</sub>
15° C.								
4.5.....	35	0	73	16	131	16	2.5	0
5.5.....	40	8	99	13	179	12	3.0	0
6.5.....	56	18	112	24	233	47	4.0	0
25° C.								
4.5.....	23	0	90	0	134	0	4.0	0
5.5.....	33	17	94	11	157	30	2.5	1
6.5.....	65	16	107	27	244	50	0	0
35° C.								
4.5.....	20	0	71	0	105	0	0	0
5.5.....	0	0	64	0	64	0	0.5	0
6.5.....	7	0	34	6	46	0	0	0

velopment of nitrate plants was superior to that of ammonium plants under corresponding conditions. Rhizome production was restricted in the ammonium-supplied plants and no significant differences in number of rhizomes as related to pH could be noted in the nitrate plants.

Green and dry weights of both three and six weeks plants were taken and showed generally the same relations as seen in the eleven weeks experiment. Differences in green and dry weights at the various temperatures were small, especially at 35° C., but a tendency prevailed for maximum weight at pH 6.5 in both nitrate and ammonium plants.

The character of the root systems varied with temperature treatment and with the form of nitrogen nutrition. At the lowest temperature the roots were large in diameter, white, and very succulent, whereas at 35° C. the roots were small, light brown, and very finely branched, forming a densely tufted system. The zone of meristematic development and elongation was very short in roots grown

TABLE 6

LEAF AND RHIZOME DEVELOPMENT OF PLANTS GROWN FOR SIX WEEKS IN SAND AT THREE SOIL TEMPERATURES AND NITRATE AND AMMONIUM NUTRITION SUPPLIED AT VARIED PH. AVERAGES BASED UPON TWO PLANTS

TEMPERATURE AND PH	PERCENTAGE INCREASE LEAF LENGTH		PERCENTAGE INCREASE LEAF NUMBER		PERCENTAGE LINEAR INCREMENT		No. OF RHIZOMES	
	NO <sub>3</sub>	NH <sub>4</sub>	NO <sub>3</sub>	NH <sub>4</sub>	NO <sub>3</sub>	NH <sub>4</sub>	NO <sub>3</sub>	NH <sub>4</sub>
15° C.								
4.5.....	90	0	227	118	530	118	18.0	1.5
5.5.....	110	18	304	135	748	179	12.0	2.0
6.5.....	64	50	547	373	937	603	17.5	9.5
25° C.								
4.5.....	100	8	500	71	1049	81	19.0	1.0
5.5.....	150	25	385	47	1118	88	18.0	1.0
6.5.....	136	41	339	272	944	421	18.5	6.0
35° C.								
4.5.....	40	8	219	0	343	8	11.0	0
5.5.....	30	8	332	8	467	12	5.5	0
6.5.....	21	30	109	39	121	81	10.0	0

at high temperatures, as shown by the presence of laterals almost to the tips of the main roots. Limited data on root tip length indicate that the greatest amount of root elongation took place at the lowest temperature.

Little difference could be noted in roots of nitrate plants with respect to pH value of the culture. Ammonium plants, however, at pH values of 4.5 and 5.5 showed a scant development of lateral roots as compared with plants grown at pH 6.5. At all temperatures the secondary laterals on ammonium plants were very short or stunted and discolored for the proximal 5-8 cm. of the newly

developed crown roots. This reduced condition of the laterals was not noted in nitrate plants.

The leaves of plants grown in this series and the former series show marked differences in degree of succulence as influenced by temperature and pH treatment. The leaves of both nitrate and ammonium plants were more succulent at low temperatures than at high ones. Nitrate plants showed little difference with respect to pH value of the culture at any one temperature, but in the case of ammonium-supplied plants, those grown at pH 6.5 were more succulent than plants grown at pH 4.5 or 5.5. This difference in succulence was more marked at 15° than at 35° C.

#### CLIPPED SERIES

To furnish information more directly applicable to turf production, an additional experiment was initiated involving the clipping of *Poa pratensis* plants under nitrate and ammonium nutrition at a uniform pH and soil temperature. The temperature tank apparatus was again used to maintain the soil temperatures at 25° C., and both nitrate and ammonium nutrition were supplied as in the previous experiments at pH 6.5. Plants were divided into three lots: clipped at 1 inch weekly, at 2 inches weekly, and control. Ten plants were used for each combination of nutrition and clipping height, and the plants were grown under greenhouse conditions from May 30 to September 14, 1936.

Clipping treatments were started on June 26 and made weekly thereafter, and the number of leaves clipped and the dry weights of the clippings were secured. Weekly counts were also taken of the total number of leaves on all clipped and control plants. Table 7 shows the percentage increase in number of green leaves for each week, based on the original number of leaves. The effects of clipping are shown in the decided reduction in the leaf production of the 1 inch series and the slight reduction in the 2 inch series, compared with the controls. Comparison of the check plants in nitrate and ammonium nutrition shows a more rapid production of leaves in ammonium nutrition throughout the experimental period, except during the first two weeks. Nitrate plants clipped at 2 inches produced more leaves than ammonium plants during the first six weeks

of the experiment, but nitrate plants clipped at 1 inch had in general a smaller production of leaves than corresponding ammonium plants throughout the experiment.

Total dry weights of the weekly clippings are shown in table 8. Nitrate-supplied cultures showed consistently higher yields than ammonium plants, although in most cases this difference was not significant. With the exception of a brief initial period in which insect infestation reduced the number of leaves, there was a consistent increase in yield of clippings over the experimental period in

TABLE 7

PERCENTAGE CUMULATIVE INCREASE OF NUMBER OF LEAVES PRODUCED  
BY PLANTS GROWN IN SAND WITH NITRATE AND AMMONIUM  
NUTRITION UNDER CLIPPED AND UNCLIPPED CONDITIONS

TREATMENT	JULY 11	JULY 18	JULY 25	AUG. 1	AUG. 8	AUG. 15	AUG. 24	SEPT. 1	SEPT. 8	SEPT. 14
Nitrate										
Check.....	10.1	33.5	71.6	92.5	129.7	150.9	181.3	204.3	219.0	243.0
1 inch.....	6.1	18.1	38.6	55.7	70.4	84.5	92.8	101.2	100.3	99.5
2 inches.....	5.0	31.9	61.3	83.4	112.4	135.8	162.9	180.0	192.4	159.6
Ammonium										
Check.....	7.4	27.5	74.2	104.7	136.9	162.1	205.2	260.1	285.0	307.2
1 inch.....	1.9	16.5	37.9	55.4	72.3	95.2	124.2	148.8	157.4	192.7
2 inches.....	.....	19.0	51.1	74.1	104.6	126.8	164.5	186.5	208.8	200.9

both nitrate and ammonium cultures, particularly in the 2 inch clipping series. Comparison of the yields obtained from the 1 inch and 2 inch series shows the 2 inch nitrate plants to have a greater yield than plants cut at 1 inch. At the end of the experiment, nitrate plants cut at 2 inches were producing three times as much as plants kept at a 1 inch height, and the total amount of clippings obtained throughout the experiment from the 2 inch series was double that for the 1 inch plants. Ammonium-supplied plants, on the contrary, produced a greater amount of clippings during the first four weeks of the experiment when kept at a 1 inch height; and during the last six weeks those plants clipped at 2 inches produced a slightly larger yield than 1 inch plants. Total weight of clippings from the 2 inch ammonium plants was only slightly larger than that obtained from the 1 inch plants.

Comparison of root and rhizome production in nitrate and ammonium-supplied plants showed in general the same relationships as in the previous experiments. Nitrate plants produced a greater number of crown roots than ammonium plants although the average length of roots was greater in the case of ammonium plants. The check plants showed the greatest number of roots and rhizomes under both forms of nutrition, and the 1 inch clipping series showed the poorest developed root systems. A greater number of rhizomes was produced under nitrate nutrition than with ammonium nitrogen.

TABLE 8

DRY WEIGHT (GRAMS) OF CLIPPINGS OBTAINED FROM PLANTS GROWN IN SAND WITH NITRATE AND AMMONIUM NUTRITION AND CLIPPED AT 1 INCH AND 2 INCH HEIGHTS AT WEEKLY INTERVALS

TREATMENT	JULY 3	JULY 11	JULY 18	JULY 25	AUG. 1	AUG. 8	AUG. 15	AUG. 24	SEPT. 1	SEPT. 8	SEPT. 14	To- TAL
Nitrate												
1 inch.....	0.362	0.310	0.183	0.283	0.392	0.371	0.504	0.619	0.660	0.463	0.492	4.64
2 inches...	.416	.327	.134	.377	.737	.800	.950	1.499	1.870	1.218	1.002	9.33
Ammonium												
1 inch.....	.335	.260	.143	.284	.305	.328	.303	.429	.516	.401	.375	3.70
2 inches...	0.243	0.229	0.116	0.240	0.369	0.348	0.401	0.583	1.096	0.697	0.594	4.92

## Discussion

### TEMPERATURE RELATIONS

If the factor of temperature is considered apart from the complex of environmental conditions under which the plants were grown, certain relations of development with temperature differences may be established. The character of the top growth varied considerably within the range of soil temperature used. Low temperatures appeared to be the most favorable both for the production of new leaves and growth in length of leaves. The type of growth resulting at 15° C. was bushy, with long, succulent leaves and numerous new leaves produced. Combined measurements of leaf length and number of leaves or linear increment show the optimum growth temperature to be about 15° C. At soil temperatures of 35° C. the plants were short and rigid, many of the leaves remaining erect

when allowed to grow uncut for three months. Bud initiation and leaf production were limited under the high temperature conditions.

Although chemical studies were not made upon the plants grown at various temperatures, the results can be interpreted partially upon the carbohydrate-nitrogen relationships, as has been done by NIGHTINGALE and others (9, 12, 13). Bluegrass plants in the present experiments, as well as those of HARRISON (5), showed the greatest amount of root and top growth at low temperatures. In both nitrate and ammonium nutrition, absorption of nitrogen took place readily enough at low temperatures so that assimilation and synthesis of protein materials resulted in pronounced increases in leaves and leaf length. High temperatures undoubtedly favored more rapid absorption of nitrogen, as indicated by pH changes in the culture solutions, but assimilation probably did not occur so readily because of the decreased carbohydrates resulting from an unfavorable photosynthesis/respiration balance. As TIEDJENS and ROBBINS (19) point out, nitrate and ammonium ions may accumulate in plants which stop active growth so that apparently the bluegrass plants grown at high temperatures were limited more by carbohydrates than by nitrogen. These implications are further borne out by the yields of top growth produced under the different temperatures and by the number of rhizomes put out by the plants. Equal weights of top growth were produced by plants grown at 15° and 25° C., whereas under soil temperatures of 35° C. the lowest weight of tops and smallest number of rhizomes were produced. Many field and greenhouse experiments with bluegrass and other plants have shown the production of rhizomes to be associated with carbohydrate storage. Under the high temperature conditions of the present experiment, carbohydrates were probably utilized in respiration faster than they were being produced, and consequently few reserves in the form of rhizomes could be established.

The character of the root systems produced was also influenced strongly by temperature. At the lowest temperature the roots of bluegrass were large in diameter, succulent, white, and with few scattered laterals, whereas at 35° C. the roots were small, light brown, and very finely branched into a dense tufted system. These observations agree with those of NIGHTINGALE (10) on apple and

peach roots. The character of the root systems seemed to be influenced by the rate of growth and maturation as evidenced by the root tip lengths. High temperatures favored rapid maturation of roots, with consequent production of laterals almost to the extreme tips of the main roots. At low temperatures more rapid elongation occurred before maturation, causing relatively long tips. The entire root systems at 15° and 25° C. were almost twice as deep as those produced at 35° C. Total root growth as measured by dry weight was equal at 15° and 25° C. and the lowest dry weights were produced at the highest temperature. HARRISON (5), working with bluegrass at temperatures of 60°, 80°, and 100° F., found that no new roots were produced with nitrate nitrogen at any of the root temperatures used over a period of two months. Essentially the same results were obtained in the present investigation, as the nitrate plants produced very few new crown roots at any temperature, whereas with ammonium nutrition many new roots were present.

#### pH RELATIONS

Under controlled nutrient conditions with nitrogen supplied in the form of nitrate, the plants differed in top and root development with varied pH, depending in part upon their age. Plants of the three weeks series showed a slight tendency for superior development at pH 6.5 at temperatures of 15° and 25°, but at 35° C. the pH 6.5 plants were decidedly inferior in linear increment to plants grown at other pH values. After six and eleven weeks growth, plants grown under similar conditions showed no significant differences with pH at the lower temperatures, but at 35° C. plants grown at pH 6.5 were slightly inferior to pH 4.5 and 5.5 plants. Under the conditions of these experiments the range of pH used apparently had little effect upon absorption and assimilation of nitrate nitrogen except at a soil temperature of 35° C. With high temperatures resulting in more active respiration, the carbohydrate reserves in these plants subjected to high temperatures were at a minimum and assimilation of the absorbed nitrogen was governed more closely by pH. Only under these conditions do the present experiments agree with those of other investigators, as for example, SPRAGUE (16), who found that bluegrass supplied with nitrate and a small amount of

ammonium sulphate produced less top growth at pH 6.5 than at pH 4.5. Root development and rhizome production in nitrate cultures were influenced by pH in the same manner as top development. Weight of roots varied little with differences in pH except at 35° C., under which condition the pH 4.5 plants produced a greater weight of roots.

Plants supplied with nitrogen in the form of ammonium at three pH values showed best development in tops, roots, and rhizomes at pH 6.5, throughout periods of three, six, and eleven weeks growth. The pH range chosen for these experiments was probably not sufficient to cover the true optimum pH for ammonium nutrition as indicated by the work of other investigators. PIRSCHLE (15), TIEDJENS (18), and TIEDJENS and ROBBINS (19) have found that high pH values allow rapid absorption and assimilation of ammonium. At low pH values ammonium ions are absorbed but little assimilation occurs, according to TIEDJENS and ROBBINS.

Rhizome production and root development in these ammonium cultures were also greatest at high pH values. Plants grown in ammonium solutions at pH 6.5 had the most extensive development of lateral roots, whereas at pH 4.5 and 5.5 there was a predominance of stunted, knobby laterals, especially near the base of the main laterals. THERON (17) and MEVIUS (7) have noted this character of roots grown in acid solutions, a result here of insufficient nitrogen assimilation for proper root development. Rhizome production, which has been found by others to occur most abundantly in plants with a plentiful supply of carbohydrates, is correlated here with a pH most favorable for ammonium assimilation, as shown by top development. At high pH values, ammonium-supplied bluegrass plants exhibited a greater vegetative condition, which would seemingly indicate a low carbohydrate reserve, facts which are not in accord with increased rhizome production.

Correlations have been found by several investigators between the amount of absorption of nitrate and ammonium ions at different pH levels and changes in the pH value of the culture media. Absorption of ammonium ions from a solution brings about an increase in acidity of the solution owing to accumulation of residual anions. Thus in the case of the bluegrass plants supplied with ammonium

at pH 6.5 it is seen that month-old plants were capable of reducing the pH of the nutrient solution to 5.0 within ten hours. The rate of absorption of ammonium, as indicated by these changes in reaction of the cultures, is governed primarily by the initial pH and secondly by temperature, greatest absorption occurring at the highest temperatures. Nitrate absorption, on the contrary, is generally slower than ammonium absorption, and there being a more equal absorption of nitrate ions and their associated cations little change in pH results. In the present experiments both nitrate and ammonium solutions applied at pH 4.5 underwent an immediate change to pH 5.0. It would seem by the extreme rapidity with which both pH 4.5 solutions changed in reaction and the difficulty encountered in leaching out the cultures to the proper value, that a part of this change in acidity may be attributed to causes other than absorption of ions by the plant roots.

#### NITROGEN NUTRITION RELATIONS

UNCLIPPED SERIES.—Within the pH range of the experiments, the plants supplied with nitrate nitrogen showed a markedly better development than those supplied with ammonium nitrogen. The top growth of nitrate plants was superior to that of ammonium plants with respect to number and length of leaves produced and in dry weight of leaves. As has been shown under the discussion of pH relations, this superiority of nitrate nutrition over ammonium nutrition in producing an extremely vegetative plant is probably a result of more rapid assimilation of nitrate within the conditions of these experiments. Absorption rates, as inferred by changes in reaction of the culture media, are indicative of a more rapid absorption of ammonium ions than nitrate ions, but actual assimilation of nitrate ions probably occurred more rapidly under the conditions of the experiments. TIEDJENS and ROBBINS (19) indicate that ammonium at high pH values is more available than nitrate, as evidenced by more rapid stem elongation, greater leaf area, and greater succulence in ammonium-supplied plants as contrasted with nitrate plants. The reactions of the culture solutions used in the present study were probably not alkaline enough to produce optimum growth with ammonium, and hence do not show the effects noted on other plants.

Rhizome and root production varied also with the type of nitrogen nutrition. The number of rhizomes per plant was significantly greater in nitrate plants than in ammonium plants, a fact which can be attributed partially to a more favorable carbohydrate balance under nitrate nutrition. HARRISON (5) also noted this difference in rhizome production under the two forms of nitrogen nutrition. Plants supplied with nitrate showed a greater dry weight of roots than corresponding plants supplied with ammonium, although the actual extent of root systems of the two types was similar. Measurements of root tip length showed more rapid elongation in nitrate plant roots at low and medium temperatures than in ammonium plants, but at 35° C. the latter excelled slightly.

CLIPPED SERIES.—The effects of clipping at 1 and 2 inch heights were studied on plants supplied with nitrate and ammonium nutrition at constant pH and temperature conditions. The plants used were transplants of low carbohydrate reserve and were placed under cutting treatment soon after transferral, facts which should be considered in the interpretation of the results obtained.

Weekly clipping yields showed a consistent increase over the eleven weeks experimental period under all conditions, but were more pronounced in the 2 inch clipping series than in the 1 inch plants. The greater yields of the 2 inch plants as compared with 1 inch may be attributed largely to the carbohydrate relations of the plants. With continuous and ample supply of nitrogen there is a pronounced tendency for vegetative growth, utilizing the available carbohydrates. In the two clipping series, the remaining leaves and stems allow a greater amount of carbohydrate to be built up in the 2 inch series, resulting in greater clipping yields. HARRISON (5) conducted a similar greenhouse study on plants supplied with plus and minus nitrate nutrition, and showed the advantages of carbohydrate reserves in minus-nitrate plants in maintaining a sustained yield on clipping to 0.75 inch height, as opposed to a low carbohydrate reserve in plants continuously supplied with nitrogen. His experiment covered approximately the same time interval as the one here reported, but with the slightly lower clipping height maintained by weekly clippings his plus-nitrate plants did not survive the experiment.

Greater yields were obtained from nitrate-supplied plants than from ammonium plants. On the basis of the previous studies over a

range of pH conditions, it may be concluded that nitrogen in the form of nitrate was more readily available even at pH 6.5 than was ammonium nitrogen, under the conditions of these experiments. Thus with a more readily available form of nitrogen, a greater stimulus was given to vegetative growth and yield with nitrate nutrition over the period in which the clipping studies were conducted.

Availability of nitrogen and relative rates of carbohydrate formation are also factors governing the production of new leaves. Weekly observations of increase in number of leaves on clipped plants showed a slightly greater production by nitrate-supplied plants during the first four to six weeks of the experiment, but during the latter part, ammonium plants produced a denser tuft with greater increase in number of leaves. The control plants initiated more leaves than either of the clipped series, and after the first two weeks of the experiment the ammonium-supplied controls showed the more rapid production of new leaves.

Comparison of these results with comparable portions of the previous experiments brings out several discrepancies with regard to optimum nutrition conditions for leaf production. Thus after three weeks duration, nitrate and ammonium plants had produced the same number of leaves in the clipping series controls, whereas in the three weeks experiment from May 4 to May 26, 1936, nitrate plants were decidedly superior. In the first eleven weeks series, nitrate plants showed almost twice as great an increase as ammonium plants, while the nitrate plants of the clipping series checks, grown under the same pH and temperature conditions, had but one-third as much leaf production as nitrate plants of the first series over the same time interval. However, the ammonium-supplied plants of both series showed almost identical production of new leaves during this time interval. These discrepancies may be due in part to the differences in atmospheric temperature conditions over the two experimental periods. As noted from the first experiment, the greatest number of leaves was produced under low soil temperature conditions, and thus the higher air temperatures prevailing during the clipping experiment played some part in reducing the leaf production of the nitrate check plants. The reasons are not clear for the similarity of leaf production of ammonium plants in the two experiments.

The number of rhizomes produced on the clipped plants was much smaller than on the control plants under both forms of nitrogen nutrition, a fact which has been observed by many workers and is correlated with the smaller amount of carbohydrate reserves established under clipped conditions.

### Summary

1. A study was made of the effects of variation in soil temperature, pH, nitrogen nutrition, and clipping height on the development of *Poa pratensis* plants grown in sand cultures under greenhouse conditions.

2. Plants grown at temperatures of 15°, 25°, and 35° C. produced at 15° C. a tall, succulent, bushy top growth with many leaves and at 35° C. an erect, non-succulent, short top growth with few leaves. Root systems of low temperature plants were large, white, succulent, and coarsely branched, while roots of high temperature plants were small in diameter, light brown, and densely tufted.

3. Plants grown with ammonium nutrition at varied pH showed best leaf, rhizome, and root development at pH 6.5, whereas nitrate plants showed little difference within a pH range of 4.5 to 6.5.

4. Nitrate plants were superior in leaf, rhizome, and root development to ammonium-supplied plants under the conditions of temperature and pH of the experiment.

5. Plants supplied with nitrate and ammonium nutrition at constant temperature and pH and clipped weekly at 1 and 2 inch heights showed greater yields with nitrate nutrition than with ammonium nutrition. Yields over a period of eleven weeks were greatest with clipping heights of 2 inches.

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## LEAF GROWTH HORMONES. I. A BIO-ASSAY AND SOURCE FOR LEAF GROWTH FACTORS<sup>1</sup>

DAVID M. BONNER, A. J. HAAGEN-SMIT, AND F. W. WENT

(WITH SIX FIGURES)

### Introduction

Few investigators have concerned themselves with a clear definition of the factors regulating leaf growth. AVERY (1), working with leaves of *Nicotiana*, was the first to correlate growth of the midrib and probably of the larger lateral veins with auxin concentration. He found the highest concentration where greatest elongation occurs. He indicated that auxin is not directly concerned in growth of the lamina, it giving no response to applied auxin. It was further pointed out by WENT and THIMANN (9) and WENT (11) that it is advantageous to distinguish between growth of the vein, which can be increased by auxin application, and growth of the mesophyll which is independent of auxin. Since growth of the latter under suitable conditions will be reflected mainly in increase in surface area, experiments on growth in leaf area may be used to analyze growth factors of the mesophyll.

Of the earlier work, that of VYVYAN (8) and of GREGORY (4) should be mentioned. VYVYAN showed that leaf growth was affected by the presence of cotyledons, and that this effect was not due to a water relationship but probably to a "food" relationship. GREGORY clearly demonstrated that leaf growth was correlated with the size of the already existing leaf area of the plant, but that this correlation could not be due to photosynthetic activity. WENT (11, 12) showed that in etiolated pea seedlings leaf growth depends upon the movement toward the growing leaves of growth factors stored in the cotyledons.

### Preliminary experiments

Considering the last fact just reviewed, pea cotyledons were chosen as a source of leaf growth factors. As it was known that a

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pea does not germinate if submerged in a small quantity of water, and that if the shoot does start to elongate no leaf growth occurs when the cotyledons remain submerged, the conclusion was drawn that the water after such a leaching might contain the active principles. This leach water, or diffusate, has the additional advantages that no toxic substances are released or undue hydrolysis occurs, as happens after making the usual plant extract (5). It was found that such a diffusate caused appreciable growth in detached growing leaf sections, and that this growth could be simulated, in part at least, by the use of sugars and amino acids as the culture solutions.

Early experiments and observations were carried out with leaves of pea seedlings, and with the leaves of *Carica papaya*. They are discussed as the preliminary steps in the development of a satisfactory quantitative test method for leaf growth regulators. The present paper describes a satisfactory quantitative bio-assay, sources, and the general nature of the action of the special substances increasing the growth rate of immature leaves.

To determine whether the factors diffusing from the cotyledons were in any manner correlated with the effect of these same cotyledons on leaf growth in the intact plant, diffusates of Daisy and Alaska peas were compared. Equal weights of peas were diffused, and the diffusates concentrated to equal volumes. They were then tested for effect on leaf growth of Little Marvel peas, which had been found to show the greatest differences in leaf size when grafted on various varieties in transplantation experiments (12). Diffusate from Daisy was found far more effective than that from Alaska, comparing well with the greater leaf growth in shoots grafted on Daisy than on Alaska peas (12).

It was also observed that growth along the edges of cut leaf strips, from the leaves of *Carica papaya*, was much greater than in the middle when such strips were floated on the solutions. A more uniform response was obtainable by perforating the leaves with a fine steel brush. Many cells are killed and wounded by such treatment, but this allows easier penetration of the solution to the intact cells in all parts of the section, resulting in uniform growth instead of growth merely along the cut edges of the section. This effect, however, is dependent upon the kind of leaf, presumably upon the perme-

ability of the lower epidermis, and will be discussed later in this paper.

Another observation was made in connection with the effect of the solutions on the parenchymatous regions of the leaf. A differential growth rate between parenchymatous and vascular tissue should result in a characteristic bulging of the tissue. When sections containing a large unstretchable vein are floated on a pea diffusate medium they show marked bulging of the intercostal regions, since the parenchyma is growing more rapidly than the veins, yet the size of the section is determined by the length of the vein. This is just the reverse of the effect obtained by auxin treatment, when the vein bulges out between the tautly stretched mesophyll. If sections are used which have small veins, however, these will be stretched by the growing mesophyll, and the size of the section is determined by the growth of the parenchyma resulting in a plane section.

### The test

In order that an object, a leaf in this case, be satisfactory for use in a quantitative bio-assay, (1) it must be reactive to the substance in question, and (2) the average of several leaves must be uniform from test to test. The following list gives the rather wide variety of leaves satisfying the first requirement, these being young rapidly growing leaves.

*Brassica oleracea*  
*Carica papaya*  
*Lactuca sativa* var. *capitata*  
*Lactuca sativa* var. *romana*  
*Ludwigia*  
*Nicotiana sylvestris*  
*Nicotiana tabacum*  
*Raphanus sativus* (French Breakfast)  
*Phaseolus vulgaris* (Kentucky Wonder)

That rapidly enlarging tissue was one criterion of sensitivity was further illustrated by determining the sensitivity of different portions of a young leaf of *Nicotiana sylvestris*. As shown in table 1, the proximal portion is the most sensitive, and AVERY (1) has shown it to be the most rapidly enlarging portion. Older leaves (6 cm. long) are found to be only slightly sensitive, or as a general rule totally insensitive.

In order to satisfy the second requirement, it must be possible to collect the leaves in such a way that the growth rate of the leaves used for different tests, and therefore of leaves collected at different times, is limited approximately to the same extent by the leaf growth factors of some standard crude extract containing leaf growth factors, as for example pea diffusate. It was early noticed that by collecting young leaves from certain pea varieties and from *Nicotiana* plants, some leaves would grow almost as well in sugar as in the pea diffusate, showing that their growth rate was limited primarily by sugar. On the other hand, some leaves grew little in

TABLE 1  
SENSITIVITY OF DIFFERENT AREAS FROM  
SINGLE LEAF OF *NICOTIANA*  
SYLVESTRIS

SECTION CUT	GROWTH IN WIDTH ABOVE IDENTICAL SECTIONS GROWN IN 1% SUCROSE SOLUTION
Apex of leaf.....	0.8 mm.
Base of leaf.....	1.4
Edge of leaf.....	0.4
Center of leaf, including midvein	0.5
Very young leaf.....	1.8

sugar but a great deal in pea diffusate, showing that their growth rate was limited by some additional factor. Such leaves obviously do not meet the second requirement just mentioned. The first foliage leaves from seedling plants, however, might be much more uniform in their sensitivity since they are dependent for their growth factors upon themselves and the cotyledons, and do not receive similar factors from older leaves. This was found to be the case in *Raphanus*, and *Nicotiana* seedlings. The first foliage leaves of seedling radish and tobacco plants have been the only such leaves worked with extensively, but from the number of plants (just listed) that respond to the pea diffusate, it might be concluded that the effect of the leaf growth substances of the pea diffusate is non-specific.

It has been found that in order to obtain leaves that may be worked with satisfactorily, the plants must be grown in the light. Leaves from etiolated pea seedlings proved too small and curled to work with, and in the case of radish there is no epicotyledonary

development in plants grown in the dark. It was attempted to cause leaves to become more deficient in leaf growth hormones by allowing the plants to remain in the dark a short period before collecting the leaves, and thereby increase their sensitivity. However, no further increase in sensitivity could be obtained by allowing plants to remain in the dark for 24 to 48 hours before collection.

*Raphanus* plants were finally selected for the leaf test, merely because large quantities of the seed were readily obtainable, making it possible to grow considerable numbers of plants. *Nicotiana* can be used equally well, as has been shown in numerous experiments. The variety French Breakfast, obtained through the Ferry Morse Seed Company of San Francisco, California, was used. The plants are grown in the greenhouse under standard conditions. When the first foliage leaves are about 50 sq. mm. in surface area they are cut from the plant, the time after germination being about 16-18 days under our conditions. It was found necessary to adhere rigidly to these conditions, since with older leaves and with second foliage leaves the uniformity of reaction is greatly diminished. Circular disks of approximately 19.5 sq. mm. in area are cut from the leaves by punching them out with a sharp cork borer. The variation in size from disk to disk is negligible. The disks are carefully washed in distilled water, and shaken for five minutes on an electric shaker to ensure thorough mixing of all of the sections. Fifteen to twenty sections are then placed in Syracuse dishes containing 2 cc. per dish of medium. This number of sections was found to be sufficient to reduce the variation in sensitivity from dish to dish so that a reproducibility of 2-3 per cent can generally be obtained. The dishes are placed in an incubator at  $25^{\circ} \pm 0.5^{\circ}$  C. for 30 hours. This length of time was found to give a maximum difference in area of about 30 per cent between control sections and sections grown in standard pea diffusate medium. Allowing the test to run for longer than 30 hours was found undesirable, since the difference in area was enhanced little and the medium becomes badly contaminated.

Several types of direct measurements were attempted for measurement of leaf growth. Leaf strips were used, their width being measured under a binocular with an eyepiece micrometer. These meas-

urements were abandoned, however, since owing to the resistance to stretching of the larger veins the growth of the sections was rather irregular. This made the personal equation too great for satisfactory quantitative work. In order to gain sufficient reproducibility it was found necessary to use a considerable number of sections, making direct measurements of area increase very laborious.

Two equally reliable methods for measuring the increased growth were therefore worked out and used: (1) direct determination of the total wet weight of the sections from one dish; and (2) measurement of the total surface area of the sections from one dish. The method of direct determination of wet weight is the faster, and therefore

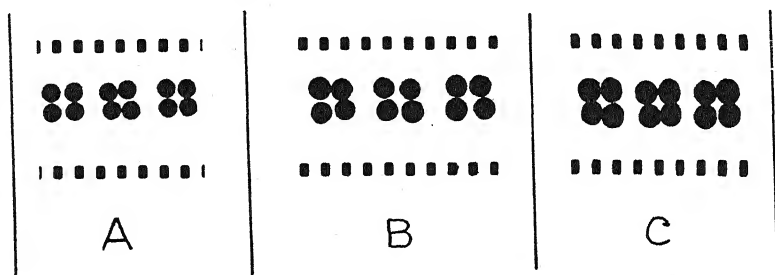


FIG. 1.—Film arrangement for determination of leaf section surface area: *a*, sections of radish leaves grown 24 hours in water; *b*, grown in 1% sucrose; *c*, grown in pea diffusate medium plus 1% sucrose.

used in routine testing, while the alternative method may be used as a check. The method of obtaining the wet weight is as follows: All the sections from a single dish are placed upon a piece of blotting paper, taking care that the sections are separated from one another. They are dried on this paper for about 1 minute, and a definite number then transferred to a piece of dry filter paper of known weight, their weight then being determined to a tenth of a milligram. These procedures are done in a uniform manner, so that the error from varying degrees of dryness appears to be negligible. For measuring the total surface area a photoelectric cell is used. The cell is arranged in such a manner that a film may be passed between it and a suitable light source directly above the cell. By such an arrangement, only light transmitted by clear areas in the film is caught by the cell. The galvanometer deflection is then a measure of the

amount of clear area on the film. Application of this principle for measurement of leaf section areas is as follows: The sections are first

TABLE 2  
COMPARISON OF ACTIVITIES MEASURED WITH PHOTO-ELECTRIC CELL, AND OF DIRECT MEASUREMENT OF FILM UNDER MICROSCOPE WITH EYE PIECE MICROMETER

SECTIONS FROM NICOTIANA LEAVES IN	METHOD	
	PHOTO-ELECTRIC CELL	DIRECT
Water.....	100	100
Sucrose (1%).....	111	110
Sucrose (1%)+pea diffusate mg. dry wt./cc.		
1.....	153	150
0.1.....	143	142

TABLE 3  
COMPARISON OF ACTIVITIES DETERMINED BY WET WEIGHT DETERMINATION, AND MEASURING SURFACE AREA WITH PHOTOELECTRIC CELL

SECTIONS FROM RAPHANUS LEAVES IN	METHOD			
	WET WEIGHT DETERMINATION		AREA DETERMINATION	
	MG./16 SECTIONS	RELATIVE TO WATER CONTROLS	GALVANOMETER DEFLECTION IN ARBITRARY UNITS	RELATIVE TO WATER CONTROLS
Water.....	42.8	100	20.3	100
Sucrose (1%).....	50.3	117	23.4	115
Sucrose (1%)+pea diffusate mg. dry wt./cc.				
1.....	63.5	149	30.8	151
0.5.....	62.2	145	30.4	149
0.2.....	61.5	144	29.6	146
0.02.....	59.2	139	29.0	143

dried as before and arranged on a piece of thin glassplate in sets of four, with lines of demarcation between the sections from different

dishes. A shadow photograph of the sections is then made on a strip of 32 mm. positive film, using a very weak exposure and an alkaline hydroquinone developer to insure maximum contrast (fig. 1). An entire experiment of 20-30 dishes may be photographed on two strips of film, so that error from varying degrees in the density of the film is minimized. In addition two or three standard areas (disks cut from a piece of steel rod) are photographed on each strip of film, so that they may be used to calibrate the measurements to the same zero point on the galvanometer for each individual film. The film is then passed over the photoelectric cell with a direct light source

TABLE 4  
EFFECT OF PH ON LEAF TEST

SECTIONS IN	PH OF MEDIUM	GROWTH RELATIVE TO SUGAR CONTROLS
Sucrose (1%).....	Unbuffered	100
Sucrose.....	4.0	100
Sucrose (1%)+pea diffusate	Unbuffered	110
mg. dry wt./cc.....		
10.....	7.0	110
10.....	6.0	111
10.....	5.0	110
10.....	4.0	109

above it. The cell catches only the light transmitted through the clear spaces on the film, these clear areas corresponding to the surface area of the original leaf sections. The galvanometer deflection is noted, and the difference between the readings is a measure of the difference in surface area of the original leaf sections. By this method the measure of activity is indicated by the increase in total surface area of twenty leaf sections grown in the crude extract over that of suitable controls. The correspondence between areas is measured in this manner, and the determination of areas by measuring the photograph under a microscope with an eyepiece micrometer is very good (table 2). The agreement between the method of determination of the wet weight of the sections and of determination of their surface area is shown in table 3.

The test is independent of pH from a pH of 4.0 to a pH of 7.0

(table 4). Below a pH of 4.0 the medium becomes toxic, owing to the external pH changing the internal sufficiently to cause damage to the tissue. The test is light independent, but is dependent on temperature, the sensitivity being less at lower temperatures.

### Sources

It has been possible to obtain extracts, active in the test just outlined, from several different sources. These fall into two general categories: (1) seed diffusates, and (2) leaf extracts.

The method of obtaining a seed diffusate is as follows:

a) Sterilization of the seed.—

1. Wash the dried seeds 10–12 times in tap water.
2. Wash 6–8 times in distilled water.
3. Place in a container from which the diffusate is to be collected (a large separatory funnel has been found excellent for this purpose).
4. Soak 2 minutes in 95 per cent alcohol.
5. Rinse 5–6 times with sterile (boiled) distilled water.
6. Soak 30 minutes in 0.1 per cent mercuric chloride.
7. Rinse six times with sterile distilled water.

b) Obtaining the diffusate:

After this treatment the seeds are covered with sterile water and left for 12 hours, then drained off and placed in fresh sterile water. The water that has been drained off is concentrated in vacuo at 30° C.

A general analysis of such a diffusate obtained from pea seeds is shown in table 5.

Pea seeds treated in this manner remain sterile indefinitely, and in the case of the leaf growth hormones, as for biotin (5), they continue to be given off at a constant rate for a very long time. Even after a month of diffusing, leaf growth hormones are given off at nearly the same concentration as when the diffusion was first started. Diffusates active in the leaf test have been obtained in a fashion similar to that just described from corn, radish, and pea seeds. It should be noted that the diffusates from these three widely different plants are all active in causing an increased growth rate in

a radish leaf. This lends additional support to the view that the active principle, or principles, in each of the three diffusates is probably the same, and that a common hormone governs the leaf growth in a large variety of plants.

TABLE 5\*

## GENERAL ANALYSIS OF PEA DIFFUSATE

FRACTION DETERMINED	PERCENTAGE DRY WEIGHT
Total nitrogen (micro-dumas).....	4.36
Total nitrogen(micro-Kjeldahl).....	3.2
Protein nitrogen.....	0.0
Non-protein nitrogen.....	3.2
Ammonia nitrogen.....	0.3
Amino nitrogen.....	1.9
Amide nitrogen.....	0.05
Polypeptide nitrogen.....	0.02
Reducing sugars.....	5.6
Sucrose.....	40.5
Non-carbohydrate reducing substances.....	10.5
Ascorbic acid.....	0.0

\* We are indebted to Prof. H. BORSOOK and Mr. W. MCRAE for the nitrogen fraction determinations.

No active extract has been obtained by a water extraction of fresh ground-up leaves. However, by placing the leaves intact in sterile water and allowing them to remain in it, at a low temperature to keep down infection, it is possible to obtain a leaf diffusate that is active. By allowing the leaves first to soak in ether for a few days, thereby inactivating any destructive enzymes, a water extract of these leaves is active in the leaf test. The extract obtained by this second method is much more active than the leaf diffusate from mature *Nicotiana* leaves. From young palm leaves (*Washingtonia filifera*) on the other hand, an exceedingly active extract may be prepared by merely allowing the leaves to soak in sterile water for a week, draining the water off, and concentrating this diffusate in vacuo. Active leaf extracts and diffusates have been prepared from leaves of *Nicotiana*, *Raphanus*, and *Washingtonia*. Yeast extract was found to be very active in the leaf test. Its activity could not be explained as due to thiamin, riboflavin, ascorbic acid, or biotin, these substances being inactive when tested by themselves.

### Units and standards

The response of leaf sections to various substances is being studied in this laboratory and further reports will be made regarding the nature and chemistry of the leaf growth hormones, but it is desirable to discuss here certain limiting factors. Sugars, as mentioned earlier in this paper, proved to be active in the leaf test, and figure 2 shows the activity concentration curve obtained for sucrose. The particu-

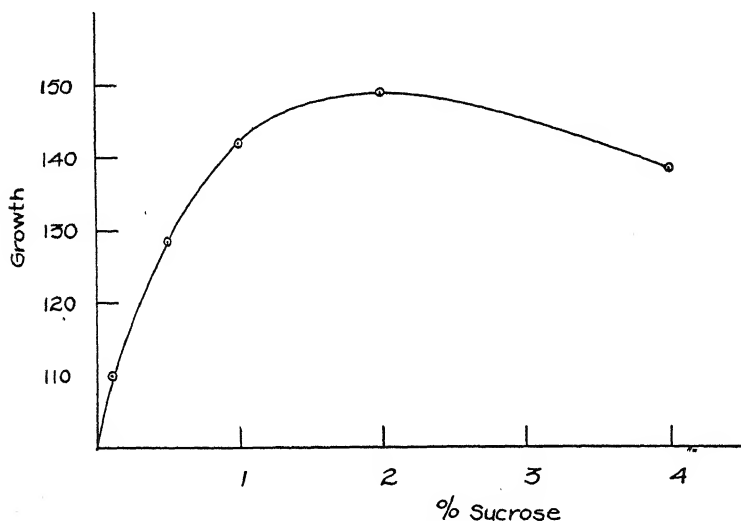


FIG. 2.—Effect of sucrose on leaf growth expressed as growth above growth of sections in water.

lar type of sugar used plays an important role. Both sucrose and glucose have the effect shown in figure 2. Mannose and galactose on the other hand give no appreciable effect. This might indicate that the effect of sugars is not merely an osmotic one. The diffusate by itself has a high activity, as shown in figure 3. To remove the possibility of a sugar being the limiting factor in the growth reaction, a basic sugar medium was added to all extracts tested and the growth compared with that of sections grown on sugar alone. The sugar selected was sucrose, it giving a good reaction (fig. 2); and as seen from table 5, pea diffusate contains 40 per cent sucrose calculated as percentage of its dry weight. Two per cent by weight of sucrose

gives optimum growth when sucrose is used by itself. By trying several dilutions it was found that in the presence of pea diffusate

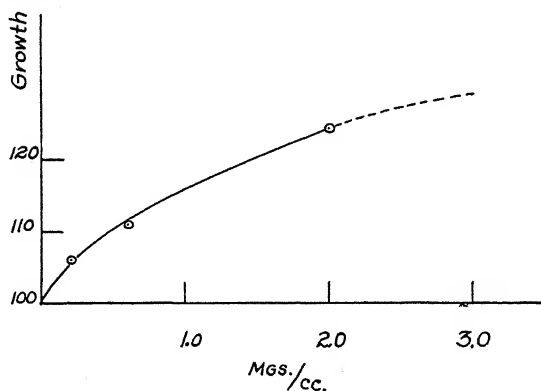


FIG. 3.—Effect of pea diffusate on leaf growth. Concentration of pea diffusate in terms of mg. dry weight of pea diffusate per cc. of water. Growth expressed as growth above growth of sections in water.

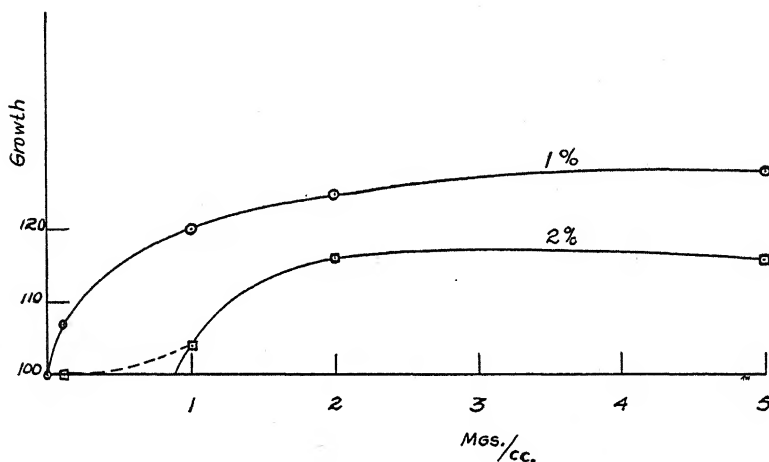


FIG. 4.—Growth of leaves in pea diffusate with different concentrations of sucrose. Growth expressed as growth above growth of sections grown in appropriate sucrose concentration.

optimum growth was obtained using a 1 per cent by weight sucrose solution (fig. 4). All extracts are now tested in a medium consisting of 1 per cent sucrose, and activity is measured in terms of the added

growth beyond that of sections grown in this basic medium. In order that activities be compared from day to day it is necessary to have a solution of "standard activity." It was found convenient to define such a solution as one containing 10 mg. dry weight of pea diffusate per cubic centimeter of 1 per cent sucrose solution. This solution is tested in a standard dilution series, consisting of four dilutions from 50 to 2 per cent (fig. 5). It has been found that if the diffusate is concentrated to a syrup and stored in the cold, standard solution

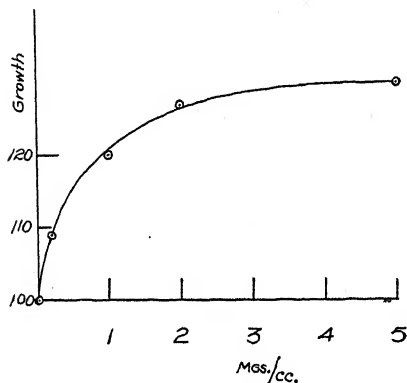


FIG. 5.—Standard dilution series. Growth expressed as growth above growth of sections grown in 1% sucrose solution.

concentrated to a syrup and stored in the cold, standard solution preparations from this concentrated diffusate give a relatively constant activity over a period of 2-3 months. The standard solution itself, however, must be made up fresh every day or two from concentrated diffusate, since after that time its activity becomes variable. That such a solution may be used as a standard is due to the fact that standard solution preparations made

from concentrated diffusates which were prepared at widely different times but from the same variety of pea have nearly the same activity.

In calculating activities of extracts it is necessary to select an arbitrary unit. We have defined one leaf unit (l.u.) as the activity of a solution containing 0.1 cc. of the standard solution; that is, 1 mg. dry weight of pea diffusate per cc. of 1 per cent sucrose solution. The number of leaf units an extract contains is determined by plotting the percentage increase of growth (growth above that of the sugar controls) against concentration in mg./cc. Graphically the concentration of the extract which shows the same activity as the standard solution at a concentration of 1 mg. dry weight of pea diffusate per cc. of 1 per cent sucrose solution is determined. This concentration gives the milligrams dry weight of the extract per leaf unit.

The preceding paragraphs have shown the effect of leaf growth

factors for a relatively short time on a leaf section. It would be desirable to determine the effect of these same factors on an intact leaf over a much longer time. For this purpose pea seedlings were grown sterily in test tubes containing nutrient medium. Twelve days after germination the foliage leaves were cut off sterily, and transferred to flasks containing suitable medium. Three series of medium were used: (1) sterile water; (2) a balanced inorganic



FIG. 6.—Growth of leaves excised from etiolated pea seedlings, cultured 1 month. Top row: in water; middle row: in inorganic salt medium plus 1% sucrose; bottom row: in inorganic salt and 1% sucrose medium, plus 1% standard pea diffusate solution.

medium (3) plus 1 per cent sucrose; and (3) the same as (2) plus 0.2 mg. pea diffusate per cc. of medium. They were allowed to grow in these media for 1 month in darkness, after which they were taken out and a shadow photograph made of them. Figure 6 indicates that over a longer period of time the effect of pea diffusate in increasing the growth of leaves is even more pronounced than in the leaf test just described. The leaves have grown more than they would have done under comparable conditions (darkness) on the plant.

#### Nature of growth response

Histological studies have been started on both *Nicotiana* and *Raphanus*. Disks were grown in a manner similar to those grown

in a regular leaf test, but were notched with regard to a known orientation of the midvein. The sections were killed with Nava-shin's solution under a slight vacuum to allow quick penetration of the fixative. They were then dehydrated in alcohol and tertiary butyl alcohol, and finally imbedded in a beeswax paraffin mixture. The sections were cut transversely and longitudinally at a thickness of  $15\ \mu$ , and were stained with a modified Flemming's triple stain.

Preliminary studies of both *Nicotiana* and *Raphanus* indicate that a gross enlargement in area and in thickness of the sections has taken place. Before killing the sections, measurements of area were made under a microscope with an eyepiece micrometer. They showed a 20 per cent increase in area of sections grown in pea diffusate over sections grown in sucrose. Measurements of thickness were made after mounting the sections. The thickness was uniformly greater (measured from epidermis to epidermis) in leaves grown in pea diffusate than in sucrose. It is of interest also that in radish and tobacco the increase in thickness was rather uniform throughout the length of the sections. This would indicate that the growth factors penetrated uniformly through the epidermis, and that in these two cases lack of penetration was not a limiting factor, as mentioned earlier for *Carica papaya*.

The histological studies also indicate a general trend toward a greater degree of cell enlargement in sections of both radish and tobacco grown in the pea diffusate than in sucrose. There was evidence not only of cell enlargement in sections grown in the crude hormone, but also very marked increase of intercellular spaces of the spongy parenchyma.

### Discussion

It has previously been demonstrated that elongation of stems is controlled by a hormone auxin (10) of known chemical constitution (6), and the growth of roots is controlled by a hormone thiamin (2, 5) of known chemical constitution (13). The present investigation indicates that the growth of leaves is also controlled by a hormone. This hormone is *non species* specific in nature, and the hormone is probably common to a considerable variety of leaves. The necessary preliminary steps in the determination of the chemical

nature of this substance (the finding of a suitable crude source of the hormone and a suitable bio-assay with which to follow its isolation) are reported in this paper. In this connection it is interesting that recently STAUFFERT (7) also has concluded that vein growth and mesophyll growth are phenomena which are differentially affected by genes. For the laciniate form of *Chelidonium majus* it was concluded that this character apparently does not reflect a morphogenetic gene but results merely in a decreased mesophyll growth.

### Summary

1. The preliminary experiments in the formulation of a bio-assay for leaf growth factors and in the finding of a crude source of these factors are described.

2. A quantitative bio-assay for hormones controlling the growth of leaves is described. The general type of leaves and the method of using them are described, together with suitable methods for measuring the increased growth of isolated leaf sections.

3. The sources of these growth factors may be classified in two general types, seed diffusates and leaf extracts. Yeast extract was also found to be a good source of these factors.

4. The basic medium of the leaf test is described as a 1 per cent sucrose solution. This is used in order that the growth rate of the leaves may not be limited by sugar. Units and standards in the leaf test are described. A solution of "standard activity" is defined as well as a "leaf unit."

5. The general nature of the growth response to pea diffusate is discussed. In both *Raphanus* and *Nicotiana* there is an increase in area of the sections grown in pea diffusate, and an increase in thickness. Cell enlargement also appears to be greater, with a separation of the spongy parenchyma and formation of more intercellular air spaces.

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# EFFECT OF VARIATION IN TEMPERATURE DURING PHOTOPERIODIC INDUCTION UPON INITIATION OF FLOWER PRIMORDIA IN BILOXI SOYBEAN

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## Introduction

Photoperiodic induction in Biloxi and other varieties of soybean is influenced greatly by factors other than photoperiod. Age of plant at the time of treatment (2), intensity of light during treatment (1), and variety of soybean used, have been shown to contribute to variations in the results obtained.

Many studies dealing with the influence of temperature upon flowering in various kinds of plants have shown that this factor, operating alone or in conjunction with photoperiod, may exert a tremendous influence upon the time and extent of flowering. But few, if any, of these studies have been directed specifically at the interrelation of temperature and photoperiod during photoperiodic induction.

In the present work on this inter-relation a careful study has been made of the extent of floral initiation following various treatments. Along with this work, studies have been made of the differences in amount of growth and change in chemical composition brought about during the period of induction.

## Literature review

GARNER and ALLARD, studying the photoperiodic response of soybeans in relation to temperature and other environmental factors, state: "The available evidence seems to indicate that under field conditions at Washington variations from year to year in date of flowering of both early and late varieties of soybeans when planted on any particular date are due chiefly to differences in temperature, while length of day is the primary external factor responsible for the fact that one variety is always relatively early and another late in

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attaining the reproductive stage" (4). CHROBOCZEK (3), working with beets, has shown that favorable conditions of light and temperature are essential to the development of a fertile inflorescence as well as to the initiation of flower primordia. By regulating the temperature and the photoperiod, the time normally required for seed-stalk formation could be greatly shortened or materially lengthened.

STEINBERG and GARNER (13) report that the critical photoperiod for flowering in soybean, *Rudbeckia bicolor*, and beet may be altered to a limited degree by temperature, and conversely, the favorable temperature range for flowering may be shifted by the action of the photoperiod.

KNOTT (7), studying the effect of temperature on the photoperiodic response of spinach, found that with photoperiod fixed at 15 hours per day seed-stalk elongation occurred sooner if temperature during treatment was held at 60° to 70° F. than it did if held at a higher or lower range. ROBERTS and STRUCKMEYER (11) found that the response of many varieties of plants to photoperiod could be materially altered by varying temperature. Although they included four varieties of soybean in their studies, the only record of their results consists of a text figure dealing with the variety Illini. This shows that flowering and fruiting on short day can be delayed by growing the plants at low temperature.

GILBERT (5) studied the interrelation of length of day and temperature in *Xanthium pennsylvanicum* and reported that temperature influenced the time of production of flower primordia. He employed both long and short photoperiod but the long photoperiod averaged only 14.6 hours. Initiation of floral primordia under GILBERT's long day condition would therefore be expected, since HAMNER and BONNER (6) report that the critical photoperiod for this species is approximately 15.5 hours. They likewise showed that temperature influences floral initiation in *Xanthium*. Both high and low temperatures applied during the 16-hour dark period inhibited initiation but had no marked influence when applied during the 8-hour photoperiod.

While this literature review presents only part of the work that has been done on the interrelationship of temperature and photoperiod, it shows that many of these studies were primarily concerned

with flowering, fruiting, or seed-stalk production, and were not restricted specifically to the role that temperature plays in the initiation of flower primordia.

### Material and methods

Biloxi soybeans were grown in the greenhouse in 4-inch pots, with the natural photoperiod extended to midnight with Mazda light of about 50 foot candles. When they were four to six weeks old they were transferred to a series of control rooms where they received photoperiodic induction at various controlled temperatures. At the time of transfer, numerous plants were dissected and, as has been found in many previous experiments, no flower primordia were present. Because the number of plants examined was large it seems safe to assume that the experimental plants were likewise vegetative at the beginning of treatment.

The induction treatments were given in four control rooms operated at 55°, 65°, 75°, and 85° F. respectively. These temperatures were controlled to within  $\pm 2^\circ$  F. Each room was illuminated with an arc light burning Sunshine carbons and furnishing more than 1000 foot candles of light at the plant surfaces. Accompanying this arc light, Mazda light of approximately 160 foot candles intensity was added simultaneously. When such an amount of Mazda light was employed in previous experiments where soybeans were grown from seed under artificial light, it was found that plants of considerably better size and quality were obtained than when the arc alone was used.

The photoperiods used were 8 hours in some experiments and 16 in others. Various temperature combinations were obtained by moving the plants from one room to another at the beginning and end of the photoperiod. Since each room was maintained at a constant temperature it was possible to obtain sixteen combinations of night and day temperature. In four of these combinations the night and day temperatures were identical; in the other twelve the night temperature differed from the day. The duration of the differential temperature treatments was three and five days in certain of the experiments and five and ten days in others. When studies of flower bud initiation were to be made, the plants were returned to the green-

house and allowed to continue their development on 16-hour photoperiods. Such a treatment when applied to the Biloxi soybean may result in the formation of flower primordia, after which the plants resume the differentiation of vegetative structures; therefore the number of flower primordia formed gives a measure of the effectiveness of the treatment applied. In experiments involving 8-hour photoperiods the plants were dissected 16 days after induction treatments were completed. The plants for chemical analyses and some of those for morphological examination were collected immediately at the end of the induction treatments.

### Experimental results with 8-hour photoperiod

#### MORPHOLOGICAL AND GROWTH RESPONSES

Plants were subjected to 8-hour photoperiods at each of sixteen combinations of temperature during the light and dark periods. For this study 480 plants were selected for uniformity from approximately 800. These were divided into thirty-two lots of fifteen plants each. Sixteen of these lots were subjected to photoperiodic induction for three days and the remainder for five. This experiment was repeated later with another set of plants that were of approximately the same stage of development at the beginning as those used in the first experiment.

Three weeks after the beginning of induction the plants were dissected. Dissection of the 480 plants of each group required about three days, but all of the plants were harvested at the same time, defoliated, and stored at low temperature with their lower ends in water until examined. As they were dissected, records were made for each plant of the total number of nodes in the main axis and the positions on the main axis of all buds containing flower primordia.

In most of the experimental lots the induction treatment was sufficient to cause differentiation of a few flower buds by most of the plants. The number of plants in each treatment that initiated flower primordia served as one index of the effectiveness of the treatment. The treatments were purposely made brief so that after return of the plants to 16-hour photoperiod the differentiation of vegetative structures would again be resumed. Since the plants were not dissected until sixteen days after completion of the induction treatments, there

was sufficient time for differentiation of many new buds, in the axils of younger leaves. In all treatments the youngest of these newly formed buds were made up of vegetative structures only. The buds containing flower primordia were located below these, and their number therefore served as an additional measure of the effectiveness of the induction treatment applied. The dissection results were analyzed by the method of analysis of variance as described by SNEDECOR (12).

The total number of plants bearing flower primordia in the first run was not significantly different from the total number in the second. There were 259 plants with flower primordia in the first and 236 in the second. There was a marked difference, however, between the lots receiving 3-day and 5-day induction periods. In the 3-day group 33 per cent of the plants initiated flower primordia, while in the 5-day group approximately 70 per cent showed floral initiation.

The most striking effect of temperature variation during induction was caused by low temperature during the dark period (table 1). When the temperature during the photoperiod was constant, fewer plants initiated flower primordia at 55° F. than at any other temperature during the dark period. The same relationship was found with respect to the total number of buds per lot containing flower primordia, mean number of buds per plant containing flower primordia, and mean number of nodes in the main axis of each plant. Temperatures during the dark period of 65° and 75° F. were most favorable so far as initiation of flower primordia was concerned. Under the conditions of the experiment, 85° F. was above the optimum for floral initiation and also for the most rapid differentiation of new nodes in the main axis.

Variations in temperature during the photoperiod also produced differences in floral initiation. With increase from 55° to 75° F. there was a trend toward increase in number of plants bearing flower primordia and number of flower primordia per plant. This was also true with respect to the total number of nodes formed.

In general the differences resulting from variation in temperature during the dark period were greater than those associated with variation in temperature during the photoperiod (table 1). These greater

TABLE 1  
EFFECT OF COMBINATIONS OF TEMPERATURE DURING 5 DAYS' IN-  
DUCTION WITH 8-HOUR PHOTOPERIODS UPON VARIOUS MORPHO-  
LOGICAL CHARACTERISTICS OF BILOXI SOYBEANS\*

TEMPERATURE DURING DARK PERIOD (°F.)	TEMPERATURE DURING PHOTOPERIOD (°F.)			
	55°	65°	75°	85°
1. TOTAL PLANTS PER LOT† BEARING FLOWER PRIMORDIA				
55°.....	0	4	10	8
65°.....	26	26	30	30
75°.....	25	30	29	29
85°.....	16	21	25	25
2. TOTAL BUDS PER LOT† CONTAINING FLOWER PRIMORDIA				
55°.....	0	5	17	9
65°.....	55	66	115	112
75°.....	65	97	123	110
85°.....	29	50	74	89
3. MEAN NUMBER OF BUDS PER PLANT‡ CONTAINING FLOWER PRIMORDIA				
55°.....	0	1.3	1.7	1.1
65°.....	2.1	2.5	3.8	3.7
75°.....	2.6	3.2	4.2	3.8
85°.....	1.8	2.4	3.0	3.6
4. MEAN NUMBER OF NODES PER PLANT IN MAIN AXIS				
55°.....	18.0	17.8	18.5	18.8
65°.....	18.3	18.6	19.0	19.1
75°.....	19.0	19.4	19.6	19.5
85°.....	18.5	18.9	18.9	19.0

\* Data based on dissections made 3 weeks after beginning of induction.

† Each lot contained 30 plants.

‡ Total buds per lot containing flower primordia divided by total plants per lot bearing flower primordia.

Differences required for odds of 19 to 1: (1) 6.2; (2) 18.0; (3) 0.8; (4) 0.5.

differences are probably to be attributed in part to the fact that the dark periods were twice as long as the photoperiods.

The differences in nodes per plant among the various treatments shown in table 1 were not great, but analysis of variance indicates that they were highly significant. The differences associated with variation in temperature during the photoperiod exceeded the 1 per cent level of significance, and those associated with differences in temperature during the dark period were found to be considerably greater. In general the treatments unfavorable to floral initiation were also unfavorable to differentiation of new nodes in the main axes of the plants. This apparent correlation suggests that the reduction in number of plants initiating flower primordia in certain treatments was brought about largely by the fact that initiation of new structures of any kind in these lots was proceeding at a very slow rate. The data of table 1, however, give the relative number of nodes present more than two weeks after the induction treatments were finished, and therefore include not only differences that arose during the induction but also any that arose subsequent to it.

Since additional data were needed to determine the extent of differences in total nodes existing immediately after induction, another experiment was performed in which dissections were made at the end of the 5-day treatment.

Special care was taken in setting up the experiment to make it as sensitive as possible, because the total number of nodes differentiated in five days would be small, making the differences between treatments correspondingly smaller. The number of temperature treatments was reduced to four to permit use of a greater number of plants per lot. Plants far in excess of the number needed in the experiment were grown on a greenhouse bench for about five weeks prior to the beginning of induction. From this supply lots of five plants each were selected, special care being taken that the individuals of each group were as nearly identical in size and stage of development as possible. The plants were labeled and one was then placed in each of the five experimental groups. This process was repeated sixty times, thereby providing five uniform lots of sixty plants each.

Four of the lots were subjected to five days' induction with 8-hour photoperiods at constant temperatures of 55°, 65°, 75°, and 85° F.

respectively. The fifth lot was dissected at the time the induction treatments of the other four were started. At the end of five days the four lots were harvested and dissected. Differences in total nodes between the initial lot and each of the other four gave a measure of the amount of differentiation that occurred in each group. The results were subjected to variance analysis and are presented in table 2.

TABLE 2  
EFFECT OF VARIOUS TEMPERATURES UPON NUMBER OF NODES  
PER PLANT DIFFERENTIATED IN MAIN AXIS DURING  
5 DAYS' INDUCTION WITH 8-HOUR PHOTOPERIODS

LOT NO.	TEMPERATURE DURING INDUCTION (°F.)		MEAN NO. OF NODES PER PLANT AT TIME OF DIS- SECTION*	MEAN GAIN IN NODES PER PLANT DURING 5 DAYS' INDUCTION
	PHOTOPERIOD	DARK PERIOD		
1.....	55°	55°	16.13	0.26
2.....	65	65	16.95	1.08
3.....	75	75	17.66	1.79
4.....	85	85	17.57	1.70
5.....	Control; no induction		15.87	.....

\* Lots 1, 2, 3, and 4 dissected at close of 5 days' induction; lot 5 at beginning of induction. Difference of 1.04 nodes per plant required for odds of 19-1.

The differences in total nodes per plant that arose between different treatments were not greatly different from those found in corresponding lots of the previous experiment. In both experiments the lowest value was obtained at 55° F. and there was an increase in number with higher temperatures. Differentiation of new structures at 55° F. was practically at a standstill during the induction period. At the most effective temperature nearly two nodes were added during the five days. The data seem to indicate therefore that the differences between lots in total number of nodes per plant (table 1) arose during the time of induction rather than subsequent to it.

When the plants used in the first dissection studies were harvested, various internodes of every plant were measured and fresh and dry weight determinations of leaves and stems of each lot were made. Analysis of length measurements indicated that the response made to treatments varied greatly from one part of the stem to another.

At the time of harvest seven separate measurements of stem length were made of each plant. The first six of these were of the first six internodes individually, the seventh included the remainder of the stem above the sixth internode. No significant differences were present in the first three internodes, which suggests that elongation in that portion of the stem was complete before the start of the experiment.

It was found that differences in temperature during the photoperiod produced significant differences in the lengths of the fourth and fifth internodes but not in the lengths of the sixth internodes and terminals. In the two former structures there was a trend toward increased length with each increase in temperature. On the other hand, temperatures during the dark period induced differences in length in each of the four uppermost regions measured. In the fourth internode greater length was associated with higher temperature. In the fifth the differences were small but in the reverse direction; longest internodes were produced at lowest temperatures. This same relationship was also found in the sixth internode but the differences were even greater. In the remainder of the stem above the sixth internode there was an increase in length for each increase in temperature during the dark period up to 75° F. At 85° F., however, the terminals were shorter than at 75° F.

These data were collected three weeks after the beginning of the induction treatments and they therefore reflect not only differences originating during induction but also such differences as developed subsequent to and as indirect effects of the induction treatments.

Data from another experiment, in which the actual gains in length made by certain internodes during the 5-day induction period were measured, indicate that these indirect effects may account for some of the differences observed. In this experiment the internodes of five plants from each of sixteen treatments were measured at the beginning of the experiment and again five days later at its termination. In these plants no significant increase in length of the first four internodes occurred during this time. The fifth internode nearly doubled in length during the five days of induction, however, and the remainder of the stem above the fifth internode more than quadrupled its length.

Although the fifth internode made considerable growth, the increases in length were fairly uniform for all lots except two, which were considerably lower than the rest. Both of these received a 55° dark period, but one was at 55° and the other at 85° F. during the photoperiod. Considerable differences occurred in length of terminals of the various lots. In almost all cases an increase in temperature during either the light or dark period resulted in increased length of this part of the stem.

In addition to the length measurements and dissection studies reported on these plants, measurements were made of changes in leaf area during induction treatment. These data were obtained by blue-printing certain young expanding leaflets at the beginning and end of a 5-day induction period. The area of these prints was later determined photoelectrically by a leaf-area measuring apparatus similar to the one described by MITCHELL (9).

At each of the four temperatures used during the photoperiod the greatest increase in area occurred when the temperature during the dark period was 75° and least when it was 55° F. At 85° somewhat smaller increases in area occurred than at 75° F. With temperature during the dark period held constant, differences in area resulting from variations in temperature during the photoperiod were not great except at 55°, when the results were consistently lower.

A study was made of the differences in fresh and dry weight at the end of a 5-day induction period of a series of plants that received the same temperature treatments as those reported in table 1. These plants were about five weeks old at the beginning of treatment. In selecting them for the experiment special care was taken to insure uniformity among the different lots. The plants were harvested at the end of the dark period following the fifth 8-hour photoperiod, and the leaves were removed from the stems. The results are shown in table 3.

The percentage of dry weight in the leaves and stems was influenced greatly by the temperature during the dark period but not by that of the photoperiod. When the temperature during the photoperiod was maintained constant, there was a progressive decrease in the percentage of dry weight with each 10° rise in temperature during the dark period. The only exception was a reversal of two of

the lots subjected to photoperiods at 85° F. The amount of floral initiation as influenced by temperature during the dark period, on

TABLE 3

EFFECT OF COMBINATIONS OF TEMPERATURE DURING 5 DAYS' INDUCTION  
WITH 8-HOUR PHOTOPERIODS UPON FRESH AND DRY WEIGHT PER  
PLANT AND PERCENTAGE OF DRY WEIGHT OF BILOXI SOYBEANS

TEMPERATURE DURING DARK PERIOD (°F.)	TEMPERATURE DURING PHOTOPERIOD (°F.)											
	LEAVES AND STEMS				LEAVES				STEMS			
	55°	65°	75°	85°	55°	65°	75°	85°	55°	65°	75°	85°
1. FRESH WEIGHT (GM. PER PLANT)												
55°.....	11.67	12.23	12.90	12.23	7.50	7.87	8.10	7.67	4.17	4.37	4.80	4.57
65°.....	12.23	13.67	14.00	14.77	7.87	8.67	8.83	9.17	4.37	5.00	5.17	5.60
75°.....	12.47	14.67	14.57	14.37	7.87	9.20	8.73	4.60	5.47	5.37	5.63	
85°.....	12.50	13.27	13.30	14.03	7.63	8.30	8.30	8.57	4.87	4.97	5.00	4.47
2. DRY WEIGHT (GM. PER PLANT)												
55°.....	1.52	1.64	1.68	1.65	1.03	1.13	1.13	1.10	0.49	0.50	0.55	0.55
65°.....	1.38	1.56	1.64	1.71	0.93	1.05	1.10	1.13	0.45	0.51	0.54	0.58
75°.....	1.37	1.60	1.61	1.55	0.92	1.07	1.07	1.00	0.46	0.54	0.54	0.55
85°.....	1.36	1.41	1.46	1.53	0.90	0.93	0.97	1.00	0.46	0.47	0.50	0.53
3. PERCENTAGE DRY WEIGHT												
55°.....	13.06	13.38	13.02	13.46	13.78	14.41	13.99	14.35	11.76	11.53	11.39	11.97
65°.....	11.31	11.41	12.74	11.60	11.86	12.12	12.45	12.36	10.31	10.20	10.52	10.36
75°.....	11.02	10.93	11.03	10.81	11.60	11.59	11.59	11.45	9.93	9.82	10.06	9.82
85°.....	10.85	10.60	11.00	10.93	11.79	11.24	11.65	11.67	9.38	9.53	9.93	9.76

Differences required for significance at odds of 19 to 1:

## 1. Fresh weight:

Leaves and stems 2.2  
Leaves..... 0.84  
Stems..... 0.46

## 2. Dry weight:

Leaves and stems 0.17  
Leaves..... 0.14  
Stems..... 0.05

## 3. Percentage dry weight:

Leaves and stems 0.38  
Leaves..... 0.47  
Stems..... 0.37

the other hand, showed a maximum at 75°. It would seem therefore that the two responses are not directly correlated.

No data were obtained as to the actual rates of photosynthesis or

respiration. It was known, however, that the dark period in which respiration predominated was twice as long as the photoperiod dur-

TABLE 4

EFFECT OF COMBINATIONS OF TEMPERATURE DURING 5 DAYS' TREATMENT  
WITH 16-HOUR PHOTOPERIODS UPON FRESH AND DRY WEIGHT  
AND PERCENTAGE OF DRY WEIGHT

TEMPERATURE DURING DARK PERIOD (°F.)	TEMPERATURES DURING PHOTOPERIOD (°F.)											
	LEAVES AND STEMS				LEAVES				STEMS			
	55°	65°	75°	85°	55°	65°	75°	85°	55°	65°	75°	85°
1. FRESH WEIGHT (GM. PER PLANT)												
55°.....	12.77	15.27	15.17	14.97	8.27	9.90	9.70	9.47	4.50	5.37	5.47	5.50
65°.....	14.27	15.47	17.47	16.30	9.27	9.97	11.17	10.20	5.00	5.50	6.30	6.10
75°.....	14.83	15.40	17.00	16.83	9.73	9.77	10.90	10.40	5.10	5.63	6.10	6.43
85°.....	14.03	15.73	16.70	16.07	9.07	10.03	10.60	10.13	4.97	5.70	6.10	5.93
2. DRY WEIGHT (GM. PER PLANT)												
55°.....	2.05	2.51	2.29	2.35	1.37	1.70	1.50	1.53	0.68	0.81	0.79	0.82
65°.....	2.13	2.30	2.41	2.25	1.42	1.53	1.57	1.43	0.72	0.77	0.85	0.82
75°.....	2.14	2.14	2.32	2.12	1.40	1.38	1.50	1.32	0.74	0.75	0.82	0.81
85°.....	1.91	2.11	2.20	2.11	1.23	1.37	1.40	1.33	0.68	0.74	0.80	0.78
3. PERCENTAGE DRY WEIGHT												
55°.....	15.77	16.46	15.10	15.70	16.53	17.17	15.46	16.20	15.11	15.16	14.45	14.85
65°.....	14.95	14.87	13.82	13.80	15.29	15.38	14.03	14.05	14.33	13.94	13.44	13.39
75°.....	14.40	13.87	13.67	12.61	14.38	14.16	13.76	12.66	14.44	13.37	13.50	12.54
85°.....	13.61	13.39	13.19	13.36	13.60	13.62	13.21	13.16	13.62	12.98	13.17	13.15

Differences required for significance at odds of 19 to 1:

## 1. Fresh weight:

Leaves and stems	0.97
Leaves.....	0.71
Stems.....	0.38

## 2. Dry weight:

Leaves and stems	0.16
Leaves.....	0.13
Stems.....	0.05

## 3. Percentage dry weight:

Leaves and stems	0.42
Leaves.....	0.64
Stems.....	0.64

ing which assimilation predominated. Increase in dry weight with decrease in temperature during the dark period was probably caused by the lower respiration at low temperature. On the other hand, the

increase in dry weight with increase in temperature during the photoperiod from 55° to 75° F. probably reflected a net increase in assimilation with rising temperature.

### Chemical composition

The carbohydrate and nitrogen metabolism of soybeans subjected to four constant temperatures and to combinations of four different temperatures during the light and dark periods has been determined. The plants were grown in the greenhouse for five weeks from time of planting. They were then divided into eight lots of sixty plants each and transferred to the control rooms previously described. Four lots were held at a constant temperature for the duration of the experiment. The other four lots were moved from one room to another at the end and the beginning of each photoperiod, so that they received different temperatures during light and dark periods. Temperature combinations during the light and dark periods were respectively 85°-55°, 75°-65°, 65°-75°, and 55°-85°. Daily photoperiods of 8 hours were applied for a period of five days. The plants were transferred from the greenhouse to the control rooms at the beginning of the photoperiod. In order to facilitate sampling, the lots of plants receiving constant temperature were moved into the control rooms one day and the lots receiving different temperatures during the light and dark periods the following day. Samples for chemical analyses were taken from representative lots each time before transferring them to the control rooms.

At the end of the dark period following the fifth short photoperiod the plants were harvested. The leaves were removed from the stems and samples for chemical analyses were prepared immediately. The method of sampling and the chemical methods employed for analyses have been reported previously (10). The only deviation was the method of determining the amount of cuprous oxide resulting from carbohydrate determinations. In these experiments the official volumetric permanganate method (8) was employed instead of the direct weight method. Results of the analyses are shown in tables 5, 6, and 7. Analyses of the two initial samples were very similar, indicating that the experimental lots were sufficiently homogeneous for the purpose of the experiment. Since intercomparison of the final com-

position of the various lots rather than net changes from beginning to end of the treatment was desired, results from these initial samples are not included in the tables. The percentage of dry weight (table 5) in the plants receiving constant temperature was greatest in the lot held at 55° F.; in those receiving alternating temperatures, greatest dry weight accumulation occurred in those receiving 55° during the dark period. Thus 16 hours of low temperature during the dark period decreased hydration of the tissue regardless of the temperature during the photoperiod.

TABLE 5

PERCENTAGE OF MOISTURE AND DRY WEIGHT AND FRESH AND DRY WEIGHT PER PLANT OF LEAVES AND STEMS OF SOYBEANS SUBJECTED TO VARIOUS TEMPERATURES DURING INDUCTION PERIOD OF FIVE 8-HOUR PHOTOPERIODS

TEMPERATURE (°F.)		PERCENTAGE MOISTURE		PERCENTAGE DRY WEIGHT		FRESH WEIGHT PER PLANT AFTER IN- DUCTION (GM.)			DRY WEIGHT PER PLANT AFTER IN- DUCTION (GM.)		
PHOTO- PERIOD	DARK PERIOD	LEAVES	STEMS	LEAVES	STEMS	LEAVES & STEMS	LEAVES	STEMS	LEAVES & STEMS	LEAVES	STEMS
85°	85°...	87.71	89.25	12.29	10.75	15.96	9.15	6.81	1.85	1.12	0.73
75	75°...	87.86	89.80	12.14	10.20	17.95	10.80	7.15	2.04	1.31	0.73
65	65°...	87.05	89.07	12.95	10.93	15.86	9.86	6.00	1.93	1.28	0.65
55	55°...	86.47	87.86	13.53	12.14	15.16	9.53	5.63	1.97	1.29	0.68
55	85°...	87.90	89.79	12.10	10.21	16.00	9.39	6.61	1.81	1.13	0.67
65	75°...	87.70	89.19	12.30	10.81	17.48	10.87	6.61	2.05	1.34	0.71
75	65°...	87.08	88.95	12.92	11.05	17.76	11.00	6.76	2.17	1.42	0.75
85	55°...	85.68	87.78	14.32	12.22	15.76	9.78	5.98	2.13	1.40	0.73

The plants that produced the greatest fresh weight (table 5) received a 75° F. temperature continuously, or during either the light or dark period. This increase in weight over the others was due primarily to an increase in the weight of the leaves. The dry weights (table 5) of the leaves and stems held at 85° during the dark period were less than those held at 55°. In the lots held at either 55° or 85° during the dark period an increase in temperature during the photoperiod resulted in an increase in dry weight. This agrees with the results shown in table 3.

The effect of temperature during the 5-day induction period on the distribution of nitrogen is shown in table 6. The percentage of

total nitrogen in the leaves and stems decreased as the temperature during the dark period decreased, regardless of the temperature during the photoperiod, and the percentage of soluble non-protein nitro-

TABLE 6

NITROGEN DISTRIBUTION IN LEAVES AND STEMS OF SOYBEANS SUBJECTED TO VARIOUS TEMPERATURES DURING INDUCTION TREATMENT OF FIVE 8-HOUR PHOTOPERIODS

TEMPERATURE (°F.)		TOTAL NITROGEN		SOLUBLE NON-PROTEIN NITROGEN		AMMONIA NITROGEN		NITRATE NITROGEN	
PHOTO- PERIOD	DARK PERIOD	LEAVES	STEMS	LEAVES	STEMS	LEAVES	STEMS	LEAVES	STEMS
PERCENTAGE DRY WEIGHT									
85°	85°...	4.736	2.322	1.326	0.986	0.008	0.037	0.089	0.223
75	75°...	4.697	2.148	0.964	0.853	0.008	0.009	0.074	0.235
65	65°...	4.081	1.716	0.865	0.677	0.007	0.009	0.038	0.155
55	55°...	3.965	1.660	0.776	0.724	0.007	0.008	0.044	0.156
55	85°...	4.549	2.501	1.265	1.529	0.024	0.029	0.090	0.225
65	75°...	4.173	1.986	0.862	1.009	0.032	0.027	0.032	0.166
75	65°...	4.092	1.787	0.797	0.824	0.015	0.018	0.038	0.153
85	55°...	3.662	1.850	0.642	0.589	0.013	0.024	0.020	0.081
MILLIGRAMS PER PLANT									
85	85°...	53.25	16.95	14.91	7.21	0.09	0.27	1.00	1.63
75	75°...	61.56	15.65	12.63	6.22	0.10	0.07	0.97	1.71
65	65°...	52.06	11.22	11.04	4.44	0.09	0.06	0.49	1.02
55	55°...	51.08	11.31	10.00	4.95	0.09	0.05	0.57	1.06
55	85°...	51.64	16.85	14.36	10.31	0.28	0.19	1.03	1.52
65	75°...	55.76	14.14	11.52	7.20	0.43	0.19	0.43	1.18
75	65°...	58.08	13.31	11.33	6.15	0.22	0.13	0.55	1.14
85	55°...	51.24	13.45	8.99	4.30	0.19	0.17	0.29	0.59

gen showed the same relationship to temperature change. The percentage of ammonia nitrogen was approximately the same for all lots of leaves receiving constant temperatures. In those lots receiving different temperatures during the light and dark periods, the ammonia nitrogen in the leaves was higher in the lots receiving low temperatures during the photoperiod. In the stems the percentage

of ammonia was uniform in all lots receiving constant temperature, except the 85°-85° lot, which was much higher than the others. In the other four lots there was little difference in the percentage of

TABLE 7

CARBOHYDRATE CONTENT OF LEAVES AND STEMS OF SOYBEANS SUBJECTED TO VARIOUS TEMPERATURES DURING INDUCTION TREATMENT OF FIVE 8-HOUR PHOTOPERIODS

TEMPERATURE (°F.)		REDUCING SUGARS		TOTAL SUGARS		SUCROSE		STARCH	
PHOTO-PERIOD	DARK PERIOD	LEAVES	STEMS	LEAVES	STEMS	LEAVES	STEMS	LEAVES	STEMS
PERCENTAGE DRY WEIGHT									
85°	85°...	0.805	0.940	1.773	1.554	0.805	0.465	1.196	0.689
75	75°...	1.079	1.511	2.061	2.109	0.816	0.451	1.302	0.755
65	65°...	2.047	4.290	2.908	4.967	0.765	0.457	3.454	1.271
55	55°...	3.854	7.471	4.907	8.459	0.820	0.675	3.362	2.594
55	85°...	0.810	1.009	0.934	1.490	0.016	0.352	1.215	1.107
65	75°...	1.253	1.685	2.017	2.481	0.602	0.648	1.765	1.212
75	65°...	1.772	3.722	2.414	4.556	0.464	0.615	2.306	1.485
85	55°...	2.960	5.978	3.889	7.272	0.733	1.031	4.301	2.481
MILLIGRAMS PER PLANT									
85	85°...	9.05	6.87	19.94	11.37	9.05	3.40	13.45	5.03
75	75°...	14.14	11.01	27.00	15.37	10.69	3.28	17.06	5.50
65	65°...	26.12	28.14	38.35	32.58	9.76	3.00	44.07	8.34
55	55°...	49.65	51.06	63.27	57.82	10.57	4.61	43.36	17.73
55	85°...	9.20	6.80	10.61	10.04	0.18	2.37	13.80	7.46
65	75°...	16.73	12.03	26.95	17.71	8.04	4.62	23.58	8.65
75	65°...	25.19	27.78	34.32	34.00	6.60	4.59	32.78	11.08
85	55°...	41.46	43.65	54.47	53.10	10.26	7.53	60.24	18.11

ammonia. The percentage of nitrate nitrogen in the leaves decreased as the temperature during the dark period was lowered, regardless of the temperature during the photoperiod. In the stems the nitrate nitrogen was higher in the 85° and 75° constant temperature lots than in the 65° and 55° lots. In the remaining four lots nitrates decreased as the temperature during the dark period decreased. All the organic and inorganic nitrogen fractions determined showed a progressive

decrease as the temperature during the dark period was lowered. While this general and uniform trend invites speculation concerning the effect of temperature on nitrogen metabolism, it is advisable to obtain more data as to the condition of the plants at the end of the light period as well as at the end of the dark period before making any generalization.

When the results were expressed as milligrams per plant the relationships were different since the size of the plant entered into the calculations. The maximum amount of total nitrogen occurred in the leaves of the lot of plants held at 75° continuously. The leaves of the two lots of plants held at 55°-55° and 55°-85° contained the same milligrams of total nitrogen, but these plants were very different in their growth habits. The former were short and sturdy while the latter were much elongated. In the stems the milligrams of total nitrogen decreased as the temperature during the dark period decreased. The actual milligrams of soluble non-protein nitrogen, ammonia, and nitrate nitrogen in both leaves and stems varied in the same manner as the percentage figures previously discussed.

When the soluble non-protein nitrogen was calculated as a percentage of the total nitrogen, the percentage in the leaves decreased as the temperature during the dark period decreased. In the stems the percentage was not very different in any of the lots when the temperature remained constant, but when the temperature was different during the light and dark periods the percentage decreased with the lower temperatures during the dark period.

The results obtained on the carbohydrate fractions are shown in table 7. The reducing sugars present in the leaves and stems were greatly influenced by the temperature during the dark period. The amount in these tissues nearly doubled with each 10° drop. The total sugar also increased as the temperature of the dark period decreased, but the magnitude of increase in the leaves was not as great for each 10° drop as it was in the stems. The amount of sucrose in the leaves was approximately the same in all the lots held at a constant temperature. When the temperatures during light and dark periods were varied, sucrose was most abundant in the lot receiving a 55° temperature during the dark, and least abundant at 85°. In the stems the amount of sucrose was high in the lots receiving a 55° dark

period, regardless of the temperature during the photoperiod; but the lot receiving an 85° photoperiod contained more sucrose than the one receiving 55° continuously. The percentage of starch in the leaves and stems increased as the temperature of the dark period decreased. The two lots of plants held continuously at 85° and 75° contained approximately the same amount of starch, and the same was true of the lots held at 65° and 55°. The former two lots, however, were much lower in starch than the latter two. In the lots that received different temperatures during light and dark there was a progressive increase in starch in both leaves and stems as the temperature during the dark period decreased. These data indicate that the plants that received a 55° dark period, regardless of the temperature during the photoperiod, were prepared to start their next photoperiod with a high concentration of carbohydrates in both leaves and stems, while the plants receiving 85° during the dark had a very much lower concentration of carbohydrates at this time. The 75° and 65° lots were intermediate to these two extremes. Thus during induction treatments the different lots attained entirely different carbohydrate composition.

The carbohydrate and nitrogen data in tables 6 and 7 show directly opposite trends with change in temperature. When the total carbohydrates were low the soluble non-protein nitrogen was high, and when total carbohydrates were higher the soluble non-protein nitrogen was low. A low temperature during the dark period resulted in carbohydrate accumulation and a decrease in soluble non-protein nitrogen, while a high temperature resulted in a reverse relationship. Consequently these lots had extremely different carbohydrate and nitrogen relationships at the termination of their 5-day induction period.

#### Experimental results with 16-hour photoperiod

The data thus far presented have shown that temperature differences applied during induction with 8-hour photoperiod bring about differences in number of flower primordia initiated. The question arises as to whether any of the temperature conditions alone might cause floral initiation even though the daily photoperiod during treatment was kept at 16 hours. To determine this point an experi-

ment was set up similar to that reported in table 1, in so far as temperature treatments were concerned, but differing in that 16-hour photoperiods were used. In this experiment half the plants were subjected to differential temperature treatments for five days and the remainder for ten, and were then returned to the greenhouse. They were harvested and dissected three weeks after the beginning of the treatments. In 480 experimental plants four were found that had initiated flower primordia. These were not associated with any particular treatment but were distributed at random among the lots, indicating that factors other than temperature were operating. The probable cause of initiation in these four cases was age of plant. They were nearly nine weeks old at the time of dissection, and it has been previously shown (10) that initiation of flower primordia on Biloxi soybeans takes place even on long photoperiod when the plants become older. The results show that the temperature treatments applied in these experiments were ineffective in causing initiation of flowers when the plants received long photoperiods during treatment.

An experiment was also conducted on 16-hour photoperiod to determine the differences in fresh and dry weight and dry weight percentages (table 4). The experimental conditions were the same as those of the experiment reported in table 3, except that a 16-hour instead of an 8-hour photoperiod was employed during the 5-day treatment.

Differences in both fresh and dry weight that were statistically significant were associated with variations in temperature of both the light and dark periods. Significant differences in percentage of dry weight, however, occurred only in response to variations in temperature during the dark period. The highest dry weight percentages were associated with the lowest temperatures, and as the temperature increased the dry weight percentages became less.

In general the fresh and dry weight data in the experiments on 8-hour and 16-hour photoperiod showed similar variations with corresponding treatments. A higher percentage of dry weight and more dry weight per plant resulted from 16-hour than from 8-hour photoperiods. This difference was to be expected since the former group

received twice as much total radiation as the latter, and the difference does not seem to have any relation to the extent of floral initiation in the two groups.

### Discussion

Temperature alone, under the conditions of these experiments, was not effective in causing floral initiation. Plants held on 16-hour photoperiod remained vegetative regardless of the combinations of temperature applied during the light and dark periods. When similar plants were subjected to 8-hour photoperiods during the application of the temperature treatments, flower buds were initiated in nearly all lots. The number of plants per lot initiating flower buds, however, and also the number of such buds formed per plant, varied considerably from one treatment to another.

It has been shown that significant differences in total nodes per plant existed among the different lots at the end of the induction treatment. The trend of these differences was similar to the trend of the differences observed in floral initiation, but their magnitude was much less. The plants of the lot kept at 75° during the photoperiod and 65° during the dark period, for example, had only 0.5 more nodes per plant than those kept at 75° during the photoperiod and 55° during the dark period, but they initiated flower primordia in 2.1 more buds per plant.

The plants at the higher temperature during the dark period were evidently initiating new nodes at a slightly greater rate than those at the lower temperature, and therefore it was expected that they would likewise be slightly more active in floral initiation. The actual increase, however, in number of plants forming flower buds and in number formed per plant at the higher temperature was far in excess of what would be expected from this cause alone.

In table 1 it was also shown that increases in temperature during the dark period from 55° to 65° and from 65° to 75° resulted in approximately equal increases in the average number of nodes formed per plant. The first 10° rise in temperature, however, resulted in a tremendously greater increase in floral initiation than the second. While the actual rate of differentiation of new structures at the growing points during induction treatments may be an important factor

in determining the effectiveness of such treatment, it seems evident from the data that other factors play an important role.

The great increase in floral initiation induced by a change in temperature during the dark period from  $55^{\circ}$  to  $65^{\circ}$  points strongly to the importance of some type of reaction occurring during this period that makes possible the initiation of flower primordia. It does not seem possible to account for the great differences in floral initiation occurring with changes in temperature during the dark period on the basis of changes in the carbohydrate and nitrogen composition. While changes of considerable magnitude were found in these fractions, their trends were unlike those found in the morphological data. In general the carbohydrates decreased and the nitrogen fractions increased with rise in temperature during the dark period. Initiation of flower primordia attained a maximum at  $65^{\circ}$  and  $75^{\circ}$  during the dark period and decreased slightly at  $85^{\circ}$ . Thus the changes in chemical composition were progressive with each  $10^{\circ}$  rise in temperature, while the morphological responses rose to a maximum and then decreased.

While changes in carbohydrate and nitrogen fractions do not seem to explain the increase in floral initiation with increase in temperature from  $55^{\circ}$  to  $65^{\circ}$  during the dark period, some chemical reaction, the nature of which is unknown, must be responsible for these increases. As measured by the floral response of the plants, the activity of such a reaction was limited at a temperature of approximately  $55^{\circ}$  during the dark period. Its activity increased sharply with increase in temperature during the dark period, the increase in the first  $10^{\circ}$  rise being very much greater than in the second. The smaller increase in activity during the second  $10^{\circ}$  rise may have been associated with the fact that the rate of differentiation of new structures by the plant imposed a limit on the rate at which flower primordia were formed.

In Biloxi soybean, conditions during the photoperiod and during the dark period can both exert a controlling influence upon floral initiation. In previous work (1) it was shown that if the light intensity was low, initiation did not occur regardless of the photoperiod employed. When intensity was high, however, initiation oc-

curred only when the photoperiod was short, indicating the necessity of the dark period. In this investigation it is further shown that when light intensity during the photoperiod is adequate, great variations in the extent of floral initiation may be induced by variations in temperature during the dark period.

### Summary

1. Biloxi soybeans were grown in the greenhouse for four to five weeks and transferred to a series of control rooms where various combinations of temperature during the photoperiod and the dark period were applied for five days. Photoperiods of both 8 and 16 hours were used.

2. The effect of these various temperatures on the initiation of flower primordia has been determined.

3. Initiation of flower primordia was influenced to a much greater extent by variation in temperature during the dark period than by variation during the photoperiod.

4. When the temperature during the photoperiod was constant, a 55° temperature during the dark period limited the amount of initiation that occurred. At 65° initiation was much more extensive. This rise of 10° in temperature during the dark period produced a greater difference in initiation than any other temperature variation of the same amount.

5. Variation in temperature resulted in the formation of different numbers of nodes. These differences, however, were not great enough to account for the differences in floral initiation.

6. Other measurements, such as fresh and dry weights, stem lengths, and leaf areas, were made. All were influenced by the different temperature treatments.

7. The carbohydrate and nitrogen content of the plants was also influenced greatly by the different temperature treatments. As the temperature during the dark period was increased the carbohydrates decreased and the nitrogen fractions increased. These changes in carbohydrate and nitrogen metabolism could not be correlated with the morphological responses.

8. None of the temperature combinations used in these experiments brought about the initiation of flower primordia when photoperiods of 16 hours were used.

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# PHOTOPERIODIC INDUCTION AS INFLUENCED BY ENVIRONMENTAL FACTORS<sup>1</sup>

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 504

ELBERT M. LONG

(WITH TWO FIGURES)

## Introduction

*Xanthium pennsylvanicum* may be photoperiodically induced by exposure to a single dark period of 15 hours at 70° F., even though subsequently grown on photoperiods not directly conducive to floral initiation. Since several lines of evidence (5) indicate that photoperiodic induction is determined by length of the dark period, it is perhaps more appropriate, with this plant, to apply the term "critical dark period" rather than "critical day" or "critical photoperiod," as has been applied to some other plants (4). Length of the critical dark period for *Xanthium* is 8½ to 11 hours, depending upon environmental factors, of which temperature seems to be the most effective.

It has been demonstrated (1, 2, 3, 5, 6, 7, 8, 9, 10, 11) that the leaves of several species of plants, including *Xanthium*, perceive the photoperiodic stimulus. Whether the leaves are the locus of photoperiodic induction is not known, but indications from the present work are that this is the case. Since the leaves perceive the photoperiodic stimulus, and since usually the stimulation occurs with exposure to 9 hours of darkness and not 8 hours, an attempt was made to determine what environmental factors influence the length of dark period necessary for induction. This seemed particularly pertinent since *Xanthium* during the winter months may respond variously, sometimes a particular treatment resulting in flowering and at other times not. The environmental factors which obviously change considerably from summer to winter in the greenhouses at Chicago are light intensity, temperature, and humidity; therefore the effect of variation in these factors upon length of the critical dark period was investigated.

<sup>1</sup> This investigation was aided in part by a grant to the University of Chicago from the Rockefeller Foundation.

### General methods

The cocklebur, *Xanthium pennsylvanicum*, was used for most of these experiments. Plants were grown from seeds collected in the vicinity of Chicago in November. Until desired for use, the fruits were stored in a bin out of doors, exposed to variations of weather. The seeds were then removed from the fruits, scratched slightly to rupture the coats, and planted within a few days in a light garden soil in ordinary flats. The seedlings were transplanted singly to pots when they had attained heights of 4-6 inches. Before the seeds germinated, the flats were placed on a well lighted bench in the greenhouse where supplementary illumination was provided by Mazda light of approximately 100 foot candles at the soil surface from sundown until 2:00 A.M. (For convenience, these conditions are referred to here as the long photoperiod.) This photoperiod varied considerably during the course of the work, but was never less than 18 hours in duration. *Xanthium* plants kept continuously under these conditions remained strictly vegetative, some for nearly a year, showing no sign of flower initiation when dissected. Biloxi soybeans were also used as experimental material. The seeds were planted in fertile soil at the rate of four per 5-inch clay pot. The pots were placed on long photoperiod and watered regularly until used for experiment.

Excessively high temperatures and low humidities were minimized by careful ventilation of the greenhouse and frequent sprinkling of the walls and benches. The plants were watered frequently with tap water, and under optimum conditions for growth, as often as twice weekly with a double strength of Shive's (12)  $R_2S_5$  nutrient solution. Unless otherwise stated, the plants grew vigorously throughout the experimental period. In general, after a given treatment the plants were returned to long photoperiod and allowed to grow until harvested.

Except when clearly evident macroscopic flowers developed, flowering response of cockleburs and soybeans was determined by careful dissection under a binocular microscope with a magnification of 20.7. Usually only the terminal buds of cocklebur were dissected, as in that species the terminal bud is almost invariably the first to initiate floral primordia, and therefore is considered the most depend-

able one in determining flowering response. With soybean it was necessary to dissect all the buds, for the terminal meristem produces floral parts only after a number of days of treatment (1), while in the vicinity of the fifth node from the terminal meristem they are differentiated 3-5 days after first being subjected to short photoperiod.

### Investigation

#### RESIDUAL EFFECT IN LEAF RESULTING FROM PHOTOPERIODIC TREATMENT

Various experiments have shown (5) that a single photoperiodically induced intact leaf of *Xanthium* may effect floral initiation in a

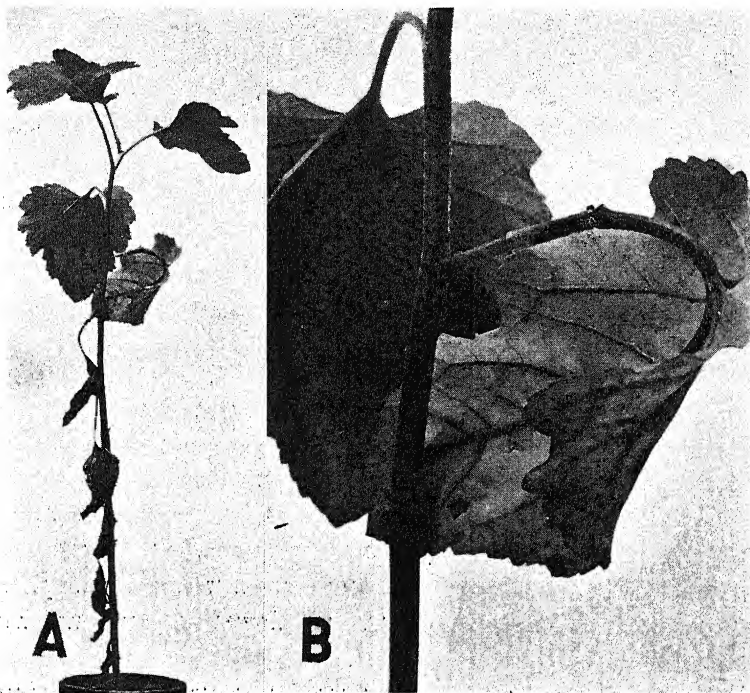


FIG. 1.—A, induced leaf grafted to vegetative plant, 4 weeks after grafting. As a result of the graft the plant has initiated floral primordia. B, enlarged view of grafted leaf.

non-induced plant when the plant is grafted to the one bearing the induced leaf. It might appear that such a leaf, after having been induced, could be removed from the plant on which it was borne and

be transferred to a vegetative non-induced plant with the result that the vegetative plant would flower because of a residual stimulus or substance transmitted to it from the leaf grafted upon it. To test this possibility, six *Xanthium* plants were subjected to two consecutive 15-hour dark periods separated by a photoperiod during which induction was completed. During the photoperiod following the second long dark period, two leaves from each of the treated plants were removed by severing the petiole obliquely across the axis about 0.5 inch from the stem. Two leaves of each of six vegetative plants which had been maintained on long photoperiod were similarly removed and the leaves from the induced plants grafted to the vegetative ones, all being subsequently maintained on long photoperiod.

TABLE 1

EFFECT OF GRAFTING LEAVES OF INDUCED PLANTS TO VEGETATIVE PLANTS

TREATMENT	NO. OF PLANTS	FLOWERING CONDITION AFTER FOUR WEEKS
Two leaves from photoperiodically induced plants grafted to each vegetative plant....	6	3 with flower primordia 3 vegetative
Two leaves from vegetative plants grafted to each vegetative plant (controls).....	6	6 vegetative

The contact was accomplished by placing one end of a short piece of glass tubing over the cut stump of the petiole of the vegetative plant and inserting the cut end of the leaf into the other end of the tube in such a way that the two cut surfaces were in intimate contact (fig. 1). The glass tube served to support the leaf and hold the cut surfaces in close contact. The plant was then placed in a nearly saturated atmosphere in a glass chamber and exposed to long photoperiod. To serve as controls for these six experimental plants, leaves from six vegetative plants were removed and grafted to six other vegetative plants in a similar manner. These control plants were maintained by the side of the experimental plants. The results are shown in table 1.

Although only three of the treated plants initiated flowers, these were the ones on which the grafted leaves remained alive for practically the entire period of the experiment (fig. 1). Most of the grafted leaves died within 10 days after grafting.

Leaves may be photoperiodically induced and subsequently may supply substances leading to floral initiation under conditions of long photoperiod or in whole plants no part of which has undergone any other type of inductive treatment.

RESIDUAL EFFECT OF TREATMENT WITH SHORT PHOTOPERIOD  
INSUFFICIENT TO BRING ABOUT PHOTOPERIODIC  
INDUCTION

In the subsequent discussion relating to subjection of plants to dark periods of varying length, unless otherwise stated a long dark period is one of 15 hours accompanied by a 9-hour photoperiod, and a short dark period one of 8 hours accompanied by a 15-hour photoperiod. Although *Xanthium* may be induced to flower when subjected to a single dark period at 70° F., as many as eight long dark periods may be required if the temperature during the dark period is 40° F. Biloxi soybean may initiate flower primordia as the result of exposure to three long dark periods alternating with short photoperiods at 70°. When more than a single long dark period is required for induction, it is possible that there may be a residual effect of each of the dark periods, which being cumulative finally results in the induced condition. In order to test such a possibility the following experiments were carried out.

On April 1, 192 vegetative *Xanthium* plants were selected for uniformity and exposed to two long dark periods at 40° F., the two periods being separated by a short photoperiod in the greenhouse at 70°. They were then returned to long photoperiod at 70°. Sixty more were selected to serve as initial controls and divided into ten lots of six plants each. One lot was exposed to one long dark period at 40° F., one lot to two long dark periods at 40°, and so on, the last lot receiving a total of ten long dark periods at 40°, each dark period being separated by a short photoperiod at 70° as previously indicated. After final treatment each lot of these initial controls was returned to the greenhouse to continue on long photoperiod until the entire experiment was harvested. Fifty other plants, selected at the same time as final controls, were divided into ten lots of five plants each and treated exactly as were the initial controls except that their

long dark period ran concurrently with that of the last group of the experimental plants.

The original 192 plants, all of which had received two long dark periods at 40° F. when returned to the long photoperiod bench, were divided into four groups of forty-eight plants each. One of these groups remained on long photoperiod for 2 days before further treat-

TABLE 2  
FLOWERING RESPONSE OF XANTHIUM PLANTS TREATED WITH  
VARIOUS NUMBERS OF 15-HOUR DARK PERIODS AT 40° F.\*

TREATMENT RECEIVED PREVIOUS TO EXPOSURE TO 15-HOUR DARK PERIODS	DATES OF LONG DARK PERIOD TREATMENTS	NO. OF 15-HOUR DARK PERIODS (AT 40°) RECEIVED SUBSEQUENT TO TREATMENT									
		1	2	3	4	5	6	7	8	9	10
No previous treatment (initial controls).....	April 4-13	6 veg	6 veg	6 veg	6 veg	6 veg	5 veg 1 fl	6 veg	6 veg	5 veg 1 fl	2 veg
Two long dark periods at 40°, then 2 short dark periods in greenhouse...	April 1 & 2; April 5-14	6 veg	6 veg	6 veg	6 veg	6 veg	6 veg	6 veg	6 veg	6 veg	6 veg
Two long dark periods at 40°, then 4 short dark periods in greenhouse...	April 1 & 2; April 7-16	6 veg	6 veg	6 veg	6 veg	6 veg	6 veg	.....	2 veg 4 fl	.....	1 veg 4 fl
Two long dark periods at 40°, then 8 short dark periods in greenhouse...	April 1 & 2; April 11-20	6 veg	6 veg	6 veg	6 veg	6 veg	5 veg 1 fl	.....	2 veg 4 fl	.....	6 fl
Two long dark periods at 40°, then 16 short dark periods in greenhouse...	April 1 & 2; April 14-23	6 veg	6 veg	6 veg	6 veg	6 veg	5 veg 1 fl	.....	5 veg 1 fl	.....	6 veg
No previous treatment, but maintained on short dark periods until April 14 (final controls).....	April 14-23	5 veg	5 veg	5 veg	5 veg	5 veg	4 veg 1 fl	2 veg 3 fl	3 veg 2 fl	3 veg 2 fl	2 veg 3 fl

\* All photoperiods and all dark periods (other than those at 40°) occur in the greenhouse.

ment, a second group remained for 4 days, a third for 8 days, and the fourth for 16 days. Each group was then subdivided into eight lots of six plants each, one lot being exposed to one long dark period at 40° F., one to two long dark periods, one to three, one to four, one to five, one to six, one to eight, and one to ten (table 2).

Although growing conditions in March, just previous to beginning the experiment, had been reasonably favorable for rapid growth, the weather from April 1 to April 20 was very cloudy. It is possible that the variable results are owing to variation in light intensity during the experimental period.

While no definite conclusions could be drawn, indications were that there was not sufficient residual effect of a long dark period at  $40^{\circ}$  in *Xanthium* to persist through two or more long photoperiods at  $70^{\circ}$ .

Another experiment was designed to show whether there is any residual effect at all, and if so, over how many long photoperiods it persists. For this experiment, beginning April 26, thirty-six pots of *Xanthium* plants were given an initial treatment of four consecutive

TABLE 3

RESPONSE OF XANTHIUM PLANTS TO INITIAL TREATMENT OF FOUR LONG DARK PERIODS AT  $40^{\circ}$  FOLLOWED BY EIGHT ADDITIONAL LONG DARK PERIODS EACH INTERSPERSED WITH VARYING NUMBERS OF SHORT DARK PERIODS AT  $70^{\circ}$  F.

NO. OF LONG DARK PERIODS OF INITIAL TREATMENT	NO. OF SHORT DARK PERIODS INTERVENING BETWEEN ADDITIONAL LONG DARK PERIODS	NO. OF PLANTS WITH FLOWER PRIMORDIA	NO. VEGETATIVE
Four	1	5	1
	2	0	6
	3	0	6
	4	0	6
	5	0	6
Four (controls)	(on continuous long photoperiod after initial treatment)	1	5

15-hour dark periods at  $40^{\circ}$ . Thereafter, six pots were returned to continuous long photoperiod as controls. Another group of six pots received, after the initial treatment, alternate short dark periods at  $70^{\circ}$  and long dark periods at  $40^{\circ}$  until they had had a total of twelve long dark periods. A third group of six got two short dark periods, a long dark period, two short dark periods, a long one, etc., until they had had a total of twelve long dark periods. The fourth, fifth, and sixth groups of six were treated in the same way except that the period of short dark period treatment was progressively one dark period longer in each. Results of this experiment (table 3) indicate that there is some cumulative effect of the long dark periods, but that it diminishes rapidly under the influence of short dark periods.

Those plants which received four long dark periods at 40°, followed by alternating short dark periods at 70° and long dark periods at 40°, produced flowers. On the other hand, none of those plants whose long dark period treatment was interrupted periodically by exposures to two or more short dark periods showed any indication of flowering. Indications are that, in *Xanthium*, a treatment with four long dark periods at 40° produces an effect in the plant, such an effect being carried over for more than 24 hours.

It might be assumed that one dark period at 40° would cause flowering if the dark period were of sufficient length. That this is very unlikely is shown by the following experiment. Forty pots of *Xanthium* were placed in the darkroom at 40° on May 9, and at more or less regular intervals four pots were removed and placed on long photoperiod in the greenhouse. None of the plants flowered even though some of them received 112 hours of continuous darkness. All plants seemed in excellent condition when removed from the darkroom. Apparently, under these conditions of low dark period temperature, photoperiodic induction does not take place unless the long dark periods are accompanied by short photoperiods.

#### EXPERIMENTS WITH BILOXI SOYBEANS

Experiments similar to those just reported were performed with soybeans. Since for induction, soybeans ordinarily require at least three long dark periods at greenhouse temperatures, it was not necessary to use the refrigerated room for dark period treatments.

On March 22, ninety-six pots, each containing four vegetative Biloxi soybean plants, were selected for uniformity from those growing on the long photoperiod bench. Excepting twenty-four pots reserved for controls, half the plants were given an initial treatment of one 15-hour dark period and half an initial treatment of two 15-hour dark periods. After the initial treatment the plants were removed to the long photoperiod bench, one-third of each group remaining there for 5 days, one-third for 10, and one-third for 20 days. After receiving the specified number of long photoperiods, one-fourth of each group received one additional long dark period; one-fourth, two long dark periods; one-fourth, three long dark periods; and one-fourth, five long dark periods. The twenty-four pots of controls were divid-

ed into two groups to serve as initial and final controls. Beginning March 22, two pots of initial controls were given one long dark period; two pots two consecutive long dark periods; two pots, three; two,

TABLE 4

FLOWERING RESPONSE OF BILOXI SOYBEANS EXPOSED TO EITHER ONE OR TWO PERIODS OF LONG DARK PERIOD TREATMENT AT DIFFERENT STAGES OF GROWTH\*

No. of LONG DARK PERIODS OF EXPOSURE IN INITIAL TREATMENT	No. of SHORT DARK PERIODS INTERVENING BETWEEN INITIAL AND FINAL TREATMENTS	DATE OF BEGINNING OF FINAL TREATMENT	No. of LONG DARK PERIODS OF FINAL TREATMENT					
			1	2	3	4	5	7
Initial controls	None . . . . .	March 22	8 veg 0 fl	7 veg 0 fl	5 veg 2 fl	2 veg 5 fl	..... 7 fl	..... 7 fl
One long dark period on March 22	5 . . . . .	March 28	7 veg	8 veg	8 veg	.....	6 fl	.....
	10 . . . . .	April 2	8 veg	8 veg	6 veg 2 fl	.....	7 fl 7 fl	.....
	20 . . . . .	April 12	7 veg	8 veg	7 veg	.....	8 fl	.....
Two long dark periods on March 22 and 23	5 . . . . .	March 29	7 veg	7 veg	6 veg 1 fl	.....	2 veg 5 fl	.....
	10 . . . . .	April 3	8 veg	7 veg	8 veg	.....	7 fl	.....
	20 . . . . .	April 13	6 veg 1 fl	7 veg 1 fl	4 veg 4 fl	.....	1 veg 6 fl	.....
Final controls	None . . . . .	April 13	8 veg	7 veg 1 fl	7 veg 1 fl	1 veg 7 fl	..... 8 fl	..... 8 fl

\* Plants grown on long photoperiod until March 22, when all were vegetative. After the various treatments all buds were carefully dissected and presence or absence of flower primordia noted. If a plant possessed one or more flower primordia it is reported as flowering (fl); other plants reported as vegetative (veg).

four; two, five; and two, seven. The final controls remained on the long photoperiod bench continuously until April 13, when they began a course of treatment similar to that of the initial controls.

After the treatments with long dark periods, all plants were returned to the long photoperiod bench where they remained for 2 weeks, after which they were carefully dissected to determine whether flowers or flower primordia were present (table 4).

As the plants grew older they required fewer long dark periods for induction. Thus the initial controls, treated March 22, showed some flowering 3 days after treatment and all flowered after 5 days. The final controls, treated April 13, showed some flowering after treatment for 2 days and all but one flowered after 4 days. This indicates that as the plants grow older they become more sensitive to photoperiodic treatment. The results show that the effect of an initial

TABLE 5  
RESPONSE OF BILOXI SOYBEANS TO INITIAL TREATMENT OF TWO  
LONG DARK PERIODS FOLLOWED BY EIGHT LONG DARK PERIODS  
EACH INTERSPERSED WITH VARYING NUMBERS OF SHORT DARK  
PERIODS

NO. OF LONG DARK PERIODS OF INITIAL TREATMENT	NO. OF SHORT DARK PERIODS INTERVENING BETWEEN ADDITIONAL LONG DARK PERIODS	NO. OF PLANTS WITH FLOWER PRIMORDIA	NO. VEGETATIVE
Two	1	0	12
	2	3*	13
	3	0	14
	4	0	10
	5	0	14
Controls	(on continuous	{	6
Two	long photo-		5
Three	period after		2
Four	initial treat-		1
Five	ment)		

\* It is possible that these plants were shaded sufficiently by others so that their supplementary illumination on the long dry bench was too low to prevent flowering.

treatment of one or two long dark periods does not persist over as many as five long photoperiods.

Another experiment was carried out to determine the possible residual effect over fewer long photoperiods. For this, twenty-eight pots were selected. Of the eight pots of controls, two received two consecutive long dark periods; two, three long dark periods; two, four; and two, five before being returned to the long photoperiod bench. The other twenty pots all received an initial treatment of two long dark periods, after which they were divided into five groups of four pots each. The first group then received a short dark period, a long dark period, a short one, and so on, until the plants had been

subjected to a total of ten long dark periods, when they were returned to the long photoperiod bench until dissected. The remaining four groups were treated in essentially the same manner except that the period of short dark periods between each of the single long dark periods of the final treatment was progressively one dark period longer for each group. The results of this experiment (table 5) unmistakably indicate that the effect of two long dark periods does not persist for more than 24 hours.

RESIDUAL EFFECT OF TREATMENT WITH SHORT PHOTOPERIOD  
SUFFICIENT TO BRING ABOUT PHOTOPERIODIC INDUCTION

Since these two experiments indicated that there was no appreciable residual effect of long dark period treatments too brief to bring about floral initiation, the question arose as to whether there was an appreciable residual effect provided the initial treatment was sufficient to cause floral initiation. In consequence, a third experiment was carried out.

On March 19, 150 pots each containing four vigorously growing, 1-month old, vegetative Biloxi soybean plants were selected for uniformity from 500 pots on the long photoperiod bench. The various treatments are given in table 6. Those plants treated with long dark periods during the period March 19-24 were placed under the diurnal photoperiod in the greenhouse at that time, without supplementary illumination. Subsequent to March 24 all plants, when exposed to long dark periods, were given dark periods of 15 hours at greenhouse temperatures.

The plants were harvested May 22, at which time eight uniform plants of the sixteen on each experimental treatment were carefully dissected and the presence of flowers at each node on the main axis noted according to the method outlined by BORTHWICK and PARKER (1). As may be seen in table 6, those plants which received the greatest number of long dark periods had the greatest number of flowers.

Treatment with long dark period from the period March 19-30 was not so effective in bringing about floral initiation as were the later periods. This may be related to the fact that the plants were relatively young during the earlier period, or it may be that the long dark periods were not so effective because they were only the natural

night lengths of that time and thus were slightly shorter than 15 hours. It may also be noted that those plants which received two

TABLE 6  
EFFECT OF VARIOUS SUCCESSIVE TREATMENTS WITH LONG DARK  
PERIOD UPON FLOWER BUD FORMATION IN BILOXI SOYBEANS

TREATMENT (PLANTS HARVESTED MAY 22)	NO. OF LONG DARK PERIODS	DATE	NO. OF PLANTS WITH FLOWER PRIMORDIA	TOTAL BUDS ON MAIN AXIS OF EIGHT PLANTS CON- TAINING FLOWER PRIMORDIA
No long dark period until March 19, then:	{ 1	March 19	0	0
	{ 3	March 19-21	0	0
	{ 5	March 19-23	7	11
	{ 6	March 19-24	7	14
	{ 7	March 19-25	8	22
	{ 9	March 19-27	8	26
Six long dark periods* March 19-25, then ten long photoperiods, March 25-April 4, then:	{ 1	April 4	7	13
	{ 3	April 4-6	7	15
	{ 5	April 4-8	8	28
	{ 7	April 4-10	8	39
	{ 9	April 4-12	8	59
No long dark period until April 1, then:	{ 1	April 1	0	0
	{ 3	April 1-3	0	0
	{ 5	April 1-5	5	14
	{ 7	April 1-7	8	31
	{ 9	April 1-9	8	44
Six long dark periods* March 19-25, then twenty long photoperiods, March 25-April 14, then:	{ 1	April 14	7	14
	{ 3	April 14-16	6	15
	{ 5	April 14-18	8	38
	{ 7	April 14-20	8	42
	{ 9	April 14-22	8	60
No long dark period until April 10, then:	{ 1	April 10	0	0
	{ 3	April 10-12	3	6
	{ 5	April 10-14	8	25
	{ 7	April 10-16	8	35
	{ 9	April 10-18	8	41

\* These long dark periods were equal to the duration of the natural night during this period and of approximately 13 hours; all other long dark periods were 15 hours.

treatments of long dark periods developed approximately as many flowers as the combined numbers produced on two separate sets of plants, one set receiving a number of long dark periods comparable

with the first treatment of the former plants and the second receiving a treatment comparable with the second treatment.

This relationship is shown more clearly in figure 2. Here three sets of plants are shown diagrammatically. Figure 2*A* shows each

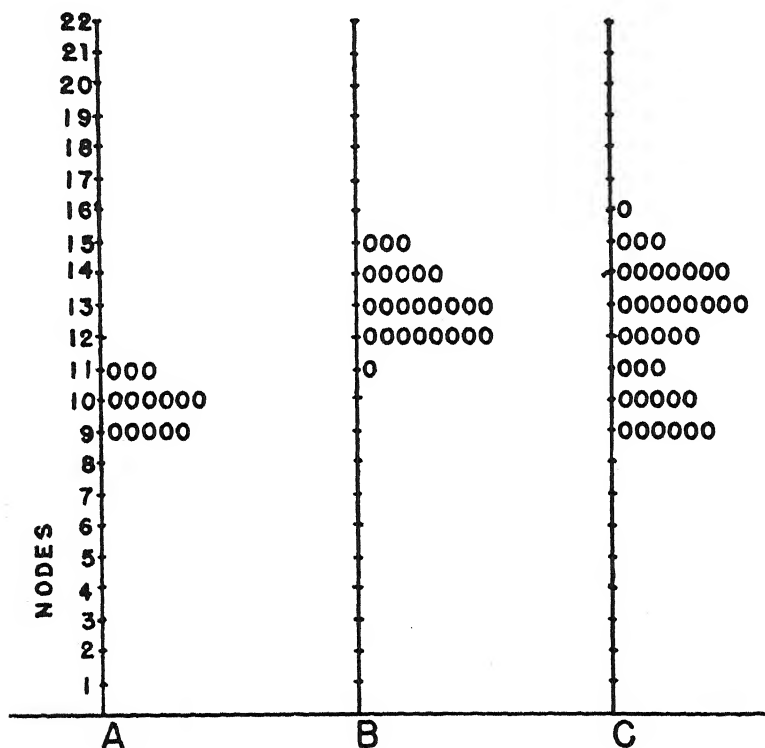


FIG. 2.—Diagrammatic representation of effect on Biloxi soybeans of various long dark period treatments.

flower of the eight plants which received six long dark periods (March 19-26). Each flower is shown in its relative position on the plant. Figure 2*B* illustrates the flowering condition of the eight plants which received five long dark periods (April 10-15). Figure 2*C* illustrates those plants which received both long dark period treatments; that is, six long dark periods (March 19-26) and five long dark periods (April 10-15). It may be seen that *C* represents almost precisely a composite picture of *A* and *B*.

These results all indicate that in soybean an induction period of several long dark periods acts more or less independently of previous treatments if the various treatments are separated by one or more short dark periods.

This is somewhat in contrast to *Xanthium* plants which are exposed to long dark periods at 40° F. In *Xanthium* the residual effect lasted 24 hours or more. In soybean also, even though a plant receives an induction period of sufficient length to bring about floral initiation, in order that more flowers be produced by a subsequent photoperiodic treatment the plant must undergo a second induction period. This is again in contrast to *Xanthium* which, once it is photoperiodically induced, seems to remain more or less permanently so and will continue to produce additional primordia over a long period of time.

#### EFFECT OF TEMPERATURE ON CRITICAL DARK PERIOD LENGTH OF XANTHIUM PLANTS OF TWO DIFFERENT AGES

Since a low dark period temperature produces a profound effect upon the length of time required for induction of *Xanthium* (5), it was decided to ascertain just what effect temperature exercised upon the critical photoperiod. Results have repeatedly been obtained in this work, and in that of HAMNER and BONNER (5), which indicate that the critical dark period is between 8 and 9 hours with summer growing conditions and dark period temperatures between 70° and 80° F. In the following experiment a determination was made of the critical dark period of plants exposed to photoperiods in the greenhouse and dark periods at 40° F.

Two hundred and forty vegetative plants were selected from among those growing on the long photoperiod bench. One-half were approximately 3 weeks old and about 6 inches in height, possessing three to five fully expanded leaves. The other half were a month older, about 1 foot in height, possessing eight to eleven fully expanded leaves. Each of these two groups was divided into twenty-four lots containing five plants each. Each lot of five young plants was combined with a corresponding lot of older plants, making twenty-five lots each of ten plants for each experimental treatment. Sixteen of these lots then received a treatment of fifteen consecutive dark

periods at 40° F. The first lot was subjected to dark periods of 8 hours, and the others to dark periods progressively longer by 20-minute increments for each lot, the sixteenth lot receiving dark periods of 13 hours. The remaining eight lots received fifteen dark periods

TABLE 7

RESPONSE OF LARGE AND SMALL XANTHIUM PLANTS SUBJECTED TO VARIOUS DARK PERIOD LENGTHS AT TEMPERATURES OF 40° AND 70°

TEMPERATURE DURING DARK PERIODS (°F.)	DURATION OF DARK PERIODS (HOURS)	LARGE PLANTS		SMALL PLANTS	
		NO. WITH IN- FLORESCENCES OR FLOWER PRIMORDIA	NO. VEGE- TATIVE	NO. WITH IN- FLORESCENCES OR FLOWER PRIMORDIA	NO. VEGE- TATIVE
40°	8.....	0	5	0	5
	8 <sup>1</sup> / <sub>2</sub> .....	1	4	0	5
	8 <sup>2</sup> / <sub>3</sub> .....	0	5	0	5
	9.....	0	5	0	5
	9 <sup>1</sup> / <sub>2</sub> .....	0	5	0	5
	9 <sup>2</sup> / <sub>3</sub> .....	1	4	0	5
	10.....	0	5	0	5
	10 <sup>1</sup> / <sub>2</sub> .....	1	4	0	5
	10 <sup>2</sup> / <sub>3</sub> .....	1	4	0	5
	11.....	2	3	1	4
	11 <sup>1</sup> / <sub>2</sub> .....	5	0	2	3
	11 <sup>2</sup> / <sub>3</sub> .....	5	0	5	0
	12.....	5	0	4	1
	12 <sup>1</sup> / <sub>2</sub> .....	5	0	5	0
	12 <sup>2</sup> / <sub>3</sub> .....	4	1	5	0
	13.....	5	0	5	0
70°	8.....	0	5	0	5
	8 <sup>1</sup> / <sub>2</sub> .....	3	2	0	5
	8 <sup>2</sup> / <sub>3</sub> .....	5	0	5	0
	9.....	5	0	5	0
	9 <sup>1</sup> / <sub>2</sub> .....	5	0	5	0
	9 <sup>2</sup> / <sub>3</sub> .....	5	0	5	0
	10.....	5	0	5	0
	10 <sup>1</sup> / <sub>2</sub> .....	5	0	5	0

at approximately 70° F. The first of these lots was also subjected to 8-hour dark periods, and the others to dark periods progressively longer by 20-minute increments for each lot, the eighth lot being exposed to dark periods of 10 hours and 20 minutes.

The results of this experiment (table 7) indicate that the critical dark period at 40° F. was about 11 hours for the large plants and at least 20 minutes longer for the small plants. Since the critical dark

period of the controls (at approximately 70° F.) proved to be about 8 hours and 20 minutes for the large and 8 hours and 40 minutes for the small plants, the lower temperature increased the critical dark period about 2 hours and 40 minutes.

These results indicate, as has other work (1), that as plants grow older they become more sensitive to photoperiodic treatment. Thus at both temperatures the old plants had a shorter critical dark period. Low dark period temperature increases the critical dark period, whether the plants are young or old, by nearly 3 hours. Thus low dark period temperature seems to produce two effects; not only must the plant receive more long dark periods at a low temperature in order to be induced, but each dark period must be relatively longer than is necessary at 70° F.

#### EFFECT OF SATURATED ATMOSPHERE ON CRITICAL DARK PERIOD OF XANTHIUM

Of the 180 vegetative plants selected from the long photoperiod for this experiment, 130 were divided into thirteen lots of ten plants each. For the duration of the experimental period of 10 days, these were maintained during photoperiods in large glass chambers in the greenhouse, where supplementary illumination was provided. Atomizers in the chambers maintained a constant fine spray of water. In order to expose the plants to a dark period of any desired duration in a saturated atmosphere, the various lots were removed from the glass chamber at timed intervals and placed in a darkroom in which the atmosphere was kept saturated. The door to the room was provided with an adequate light and air trap. At 7:30 P.M. one lot of ten plants was removed from the glass chamber and placed in the darkroom. At 7:50 P.M. another lot was placed in the darkroom; at 8:10, another, and so on until 11:30 P.M. The whole group of 130 plants was returned to the glass chamber at 7:30 A.M. Thus the various lots received dark periods varying by 20-minute increments from 8 to 12 hours. All transfers were made as rapidly as possible.

Several determinations of the relative humidity in the darkroom were made with a psychrometer, and no wet bulb depression was observed. As far as is known, all leaves were dripping wet throughout the experimental period, except for two occasions when brief

stoppages in the atomizers in one of the glass chambers permitted the humidity to fall for a short time during two photoperiods.

The remaining fifty plants served as controls. They were divided into five lots which were left on the long photoperiod bench in the greenhouse during the photoperiods of the experimental period, and subjected to ten dark periods in an ordinary darkroom at night.

TABLE 8  
RESPONSE OF *XANTHIUM* PLANTS IN SATURATED ATMOSPHERE  
AND AT NORMAL GREENHOUSE HUMIDITIES TO  
VARIOUS DARK PERIODS

ATMOSPHERIC CONDITION	LENGTH OF DARK PERIOD (HOURS)	NO. WITH IN- FLORESCENCES OR FLOWER PRIMORDIA	NO. VEGETATIVE
Saturated or nearly so	8	0	10
	8 $\frac{1}{2}$	0	10
	8 $\frac{2}{3}$	1	9
	9	8	2
	9 $\frac{1}{2}$	9	1
	9 $\frac{2}{3}$	10	0
	10	10	0
	10 $\frac{1}{2}$	10	0
	10 $\frac{2}{3}$	10	0
	11	10	0
	11 $\frac{1}{2}$	10	0
	11 $\frac{2}{3}$	10	0
	12	10	0
Normal greenhouse humidities	8	0	10
	8 $\frac{1}{2}$	0	10
	8 $\frac{2}{3}$	10	0
	9	10	0
	9 $\frac{1}{2}$	10	0

Dark periods were of 8 hours' duration for the first lot, and progressively longer by 20-minute increments for each of the others.

The results (table 8) indicate that very high humidities have only a minor effect on critical day length of *Xanthium*. Other experiments with low humidities (10-18 per cent) indicated similar results.

#### EFFECT OF LOW LIGHT INTENSITY ON CRITICAL DARK PERIOD OF *XANTHIUM*

Because of the somewhat irregular responses noticed at times with plants growing in the greenhouse in midwinter, it seemed possible

that low light intensity might affect critical dark period in *Xanthium*. To investigate this possibility, 180 plants were selected from those on long photoperiod. Fifty were left under full illumination as controls, and 130 were placed under a framework covered with several layers of cheesecloth, two layers of which had been tinted a light silver gray color. Light intensity was measured with a Weston light

TABLE 9  
FLOWERING RESPONSE OF XANTHIUM PLANTS SUBJECTED TO  
PHOTOPERIODS AT HIGH OR AT LOW LIGHT INTENSITIES TO  
DARK PERIODS OF VARIOUS LENGTHS

APPROXIMATE LIGHT INTENSITY IN FOOT CANDLES DURING PHOTOPERIODS OF EXPERIMENT	DARK PERIOD DUR- ING EXPERIMENT (HOURS)	NO. WITH IN- FLORESCENCES OR FLOWER PRIMORDIA	NO. VEGETATIVE
550	8	0	10
	8½	0	10
	8¾	2	8
	9	6	4
	9½	10	0
	9¾	10	0
	10	10	0
	10½	10	0
	10¾	10	0
	11	10	0
	11½	10	0
	11¾	10	0
	12	10	0
5500	8	0	10
	8½	9	1
	8¾	10	0
	9	10	0
	9½	10	0

meter, and at 11:00 A.M. on a clear day was found to vary at leaf surface from 500-600 foot candles on the shaded plants to 5000-6000 foot candles on the control plants. Mazda lamps were included under the cheesecloth so that the supplementary illumination was of the same duration and approximately the same intensity for experimental and control plants.

After 10 days of shading, when the plants were becoming slender and spindling, the critical dark period was determined for both groups. They were divided into lots of ten plants each, and the ex-

perimental plants given ten dark periods which varied in duration by 20-minute increments from 8 up to 12 hours. Controls were also divided into lots of ten plants each and also received ten dark periods in the same darkroom, which was maintained at approximately 70° F. The dark periods for the various lots of controls varied in duration by 20-minute increments from 8 up to 9½ hours. After the treatment all lots, both experimental and controls, were again subdivided, half of each lot placed under the cheesecloth to develop in the shade and the other half allowed to develop under full illumination. All were dissected 2 weeks after the end of the experimental period. Although flowers developed more rapidly in those plants subjected to full illumination after photoperiodic treatment, light intensity subsequent to photoperiodic treatment did not affect the critical dark period, so the last subdivision is not indicated in table 9, where the results of this experiment are reported.

As shown in table 9, the critical dark period of the shaded plants is between 8½ and 9½ hours, but is difficult to determine precisely, since with dark periods of 8½ and 9 hours some of the plants flowered and others did not. The plants grown in full sunlight had a critical dark period between 8 and 8½ hours. Thus plants grown in shade have a somewhat longer critical dark period than plants grown in full sunlight.

### Summary

1. The leaves of *Xanthium* plants are the locus of photoperiodic induction. Induced leaves, when grafted to vegetative plants, continue to supply a stimulus for flower initiation when exposed to continuous long photoperiod.

2. If during each 24-hour period, *Xanthium* plants are exposed to 15-hour dark periods at 40° F. alternating with 9-hour photoperiods at approximately 70°, then six to eight such consecutive cycles of dark period and photoperiod are required for photoperiodic induction. A treatment with four such cycles produces a residual effect which lasts more than 24 hours.

3. In these experiments, Biloxi soybeans flowered only after exposure to consecutive long dark periods, each alternating with a short photoperiod. Plants did not flower, regardless of the number of long

dark periods they received, unless at least three of the dark periods were in consecutive order.

4. Soybeans exposed to an induction period of three or more consecutive long dark periods, and which produced as a result a certain number of flower primordia, would not produce new primordia subsequently unless they were again exposed to another induction period of three or more long dark periods.

5. The length of the critical dark period for *Xanthium* is not constant. It decreases slightly in length with increasing age of the plant and varies slightly with variations in humidity. If plants are shaded during their photoperiods their critical dark periods increase slightly in length.

6. Variations in temperature greatly affect the length of the critical dark period, that of plants at 70° F. being approximately  $8\frac{1}{2}$  hours while that of plants at 40° is approximately 11 hours.

This investigation was carried on under the direction of and in association with Dr. KARL C. HAMNER, Department of Botany, University of Chicago.

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# STRUCTURE OF THE SHOOT APEX IN ZAMIA<sup>1</sup>

MARION A. JOHNSON

(WITH ELEVEN FIGURES)

## Introduction

In a recent paper on the shoot apex of *Ginkgo biloba* L., FOSTER (4) has described a structural organization unique for meristems among vascular plants. The essential feature is a centrally placed group of enlarged cells whose origin is traced to several superficial initials occupying the apex of the shoot. These central cells, characterized by their large size, irregular arrangement, large nuclei, and much vacuolated cytoplasm, give rise to the remainder of the shoot apex, which radiates from them as a flanking and rib meristem. FOSTER's suggestion that it would be of interest to explore the situation in the cycads was immediately acted upon by the writer, who had material of *Zamia integrifolia* Ait. and *Z. umbrosa* Small in process of preparation for a study of the origin and development of the leaf tissues. It was soon apparent that a close similarity existed between the fundamental architecture of the shoot meristem in *Zamia* and in *Ginkgo*.

CHAMBERLAIN'S (1) recent volume on gymnosperms reveals that little attention has been devoted to the structure and development of the shoot meristem in cycads. HOFMEISTER (6) in 1857 reported a three-sided apical cell for *Zamia longifolia* (now regarded as a synonym for *Encephalartos longifolius* Lehm.). His figure, however, taken from a transverse section through the apex, is not convincing. Later (7) he grouped the cycads, in a comparison based on the shoot meristems, with those plants having either dolabrate or inverted pyramidal apical cells. STRASBURGER (13) questioned HOFMEISTER'S observations and stated that he was unable to discover an apical cell in *Cycas revoluta*. He compared the meristem of *Cycas* with that of the conifers in general, noting in particular the poor de-

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marcation between dermatogen and periblem. He concluded that the relationship between the cryptogamic (with apical cells) and the coniferous type was not to be sought in the cycads but rather in *Lycopodium*. The need for an examination into the meristem of the cycads seemed evident, and it was with this in mind that the present investigation was undertaken.

### Material and methods

The data recorded in this paper were obtained from four lots of material collected and fixed as follows. Lot I consisted of about two dozen stem tips of *Zamia integrifolia* and *Z. silvicola* Small, obtained in the vicinity of Floral City, Florida, January 22, 1938, by my colleague Dr. M. A. CHRYSLER. Fixation was in formalin-acetic alcohol. Lot II contained two apices from plants growing in the Rutgers University greenhouse, fixed January 13, 1939, in Navashin's solution. Lot III was collected by the writer in Florida on March 26 to 28, 1939, and included: (1) *Z. umbrosa* from Daytona; (2) *Z. integrifolia* from the region around Fort Lauderdale, in all stages of leaf development, also plants with mature microsporangiate cones and others with megasporangiate cones containing female gametophytes with mature archegonia; (3) *Z. silvicola* from the Hattie Bauer hammock in the vicinity of Miami. The apices were fixed either in the field or during the evening following collection, after having been carefully dissected from the inclosing leaf bases and trimmed to a base 5 mm. square. Satisfactory results were obtained from the use of Navashin's solution, formalin-acetic alcohol and P.F.A.<sub>3</sub> (Allen's modification of Bouin's fluid). Lot IV was made up of seeds of *Z. integrifolia* in several stages of germination; fixation was in Navashin's solution. All material was imbedded in paraffin by the tertiary butyl alcohol method as outlined by JOHANSEN (8). This treatment facilitated the sectioning of tissues heavily stored with starch.

The drawings have been made with the aid of a Zeiss drawing apparatus, and with the exception of figures 7, 10, and 11 have been reduced from an original magnification of  $\times 430$ .

Heidenhain's iron-alum haematoxylin, safranin fast-green, and the tannic acid and iron chloride method as recommended by FOSTER (3) were satisfactory for staining.

### Investigation

#### ORGANIZATION OF MERISTEM

The short tuberous caudex of the three species studied is terminated by a massive, gently rounded cone or mound of meristematic tissue which is adequately protected by encircling, spirally arranged leaves and scale leaves in varying stages of development. The size and inclination of the sides of the cone are correlated with the stage of development at which the plants are examined. In mature embryos the diameter of the tip  $50\ \mu$  from the apex measures about  $200\ \mu$ , while in well grown cone-bearing specimens diameters of  $860$  and  $625\ \mu$  at  $156$  and  $78\ \mu$  respectively from the apex have been observed. These large meristems have gently sloping sides, are mound-like rather than conelike in shape, and may attain a depth of  $460\ \mu$  from apex to base of the central zone. The dimensions most commonly observed were a diameter of about  $400\ \mu$  at  $78\ \mu$  from the apex and a depth of  $300\ \mu$ .

The cellular arrangement within the massive apical meristem constitutes four distinct zones, each of which plays a definite role in the development of the mature stem. The relationship between these regions is shown diagrammatically in figure 1. Comparison of this figure with that given by FOSTER (4) for *Ginkgo biloba* shows that the same fundamental plan occurs in both *Zamia* and *Ginkgo*. A clearer understanding of the organization of the meristem as a whole can perhaps best be gained through a brief description of each zone, before a more detailed treatment is attempted.

The apex of the shoot is occupied by a superficial group of cells, the apical initials (zone I), which owing to the number and method of division are not to be confused with the familiar apical cells of the cryptogams. These initials divide by periclinal and anticlinal walls and eventually give rise to all the cells of the meristem. Their immediate progeny, chiefly from periclinal division, accumulate in the center of the meristem as a conspicuous central core (zone II). From the standpoint of origin, position, and relationship to the remainder of the apex, zone II is a distinct unit, but when considered on the basis of function it must be separated into two regions—IIa and IIb. Longitudinal sections show the cells in the upper portion (IIa) to be

relatively small, with dense cytoplasm and heavily stained nuclei; in contrast, those in II*b* are generally larger, with highly vacuolated cytoplasm and faintly stained nuclei. It is evident that in II*a* the principal activity is cell propagation while in II*b* it is cell enlargement.

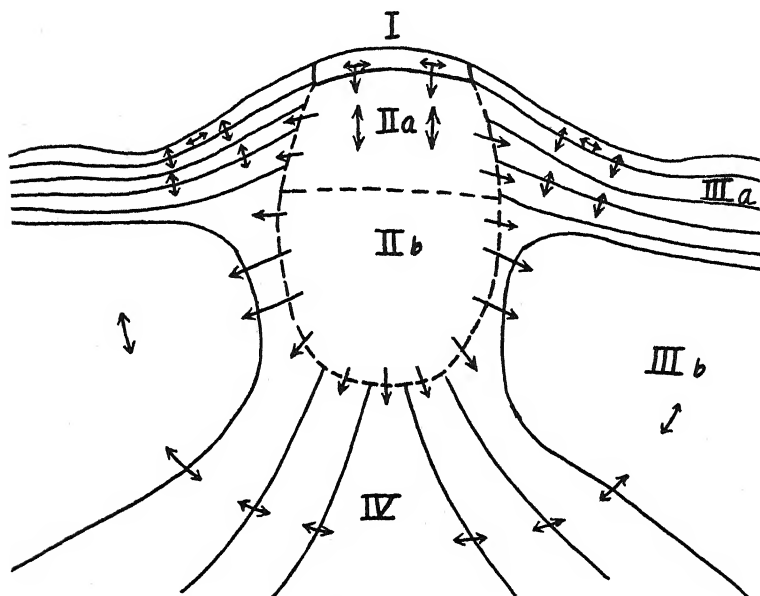


FIG. 1.\*—Diagram showing zonal nature of shoot apex of *Zamia* in median longitudinal section. Zone I indicates position of apical initial group. Zone II, which originates from apical initials, represents central mother cells whose upper members (II*a*) are actively dividing while those below (II*b*) are generally in some phase of enlargement. Zone III constitutes the flanking peripheral layers. Zone IV is a rib meristem from which the pith is differentiated. Note importance of central mother cells (zone II) in contributing to remainder of meristem. Arrows indicate general direction of growth in various zones.

\* Legends for drawings: *a*, apical initials; *p*, periclinal divisions in surface layers; *pl*, peripheral layers (zone III); II*a*, actively dividing central mother cells; II*b*, enlarging central mother cells; *ppl*, periclinal divisions in cells derived from surface layer; *r*, rib meristem; "wall thickenings" in black.

It should be emphasized that zone II is the most characteristic feature of the entire meristem. FOSTER (4) has accordingly proposed that it be known as the central mother cell zone in *Ginkgo*. His term is equally appropriate for *Zamia*, since here also both zones III and IV show by their radial cellular alignment that their origin for the

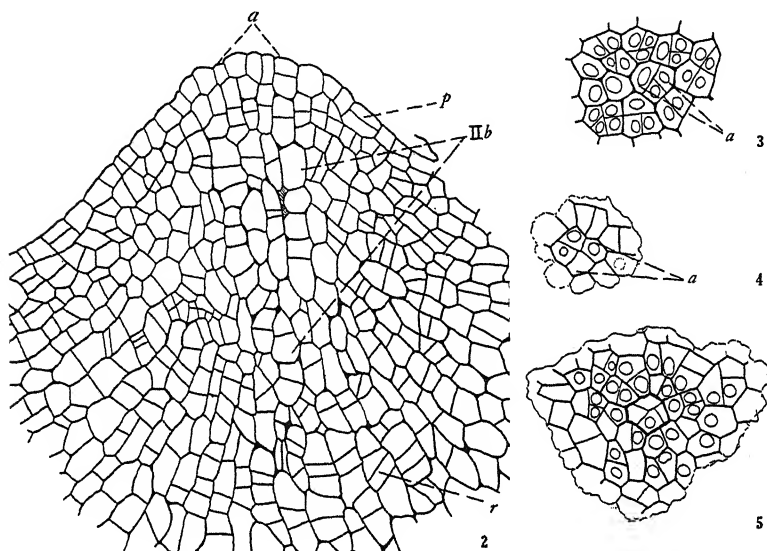
most part can be properly traced to this central region. Zone III is a flanking meristem surrounding the central mother cells, and ultimately matures into epidermis, cortex, vascular tissue, and probably the outer portion of the pith. Zone IV, on the other hand, consists of a shallow rib meristem underlying the central mother cells. It soon differentiates into the remainder of the conspicuous pith.

#### ZONE I: APICAL INITIALS

Determination of the exact location of an apical cell group must of necessity depend upon a study of median longitudinal and transverse sections. Considerable difficulty was met in this respect. In *Zamia* the apex is low and broad; in figures 2, 6, and 8 diameters of 150, 175, and 250  $\mu$  respectively are found 30  $\mu$  from the apex. Therefore, longitudinal series cut 8  $\mu$  in thickness show five or six sections having a similar structure, any one of which might be identified as median. Great care was exercised to make certain that each section used for drawings was truly median. Figures 2, 6, and 8 show that the apex of each shoot is covered with a single layer of cells sometimes somewhat larger than those flanking them. Their number is highly variable; for example, in conical apices (figs. 2, 6) there are probably six to eight respectively in each median section. If these tips are symmetrical in shape, as is usually the case, the entire number would be fifty or more. Broad mound-shaped tips similar to that in figure 8 (and which may be much larger in old specimens of *Z. integrifolia*) must approach one hundred. On the other hand, in an embryo (fig. 7) the number probably will be under a dozen. The exact center of the tip, if it were possible to locate it with certainty, would probably be occupied by a cell capable of forming a tetrad similar to that shown in figure 4. Figure 5, the upper surface of which is just 8  $\mu$  below figure 4, indicates that the base of the superficial tetrad is in the center of the figure and surrounded by some forty cells dividing in a similar manner. These are the initial cells as viewed in transverse section, and owing to their method of cleavage, form an irregular, cellular pattern clothing the summit of the shoot. While these cells are more or less identical in size, content, and method of cleavage at a given time, it is only the ones at the exact apex that continue to supply cells throughout the life of the

plant and which are thus the true apical initials. Those on the periphery are gradually crowded to the outside and eventually become engulfed in the production of leaf primordia.

In view of the fact that HOFMEISTER (7) considered the cycads to have either dolabrate or pyramidal apical cells, it might be wise to examine the method of segmentation in the apical initial group. It



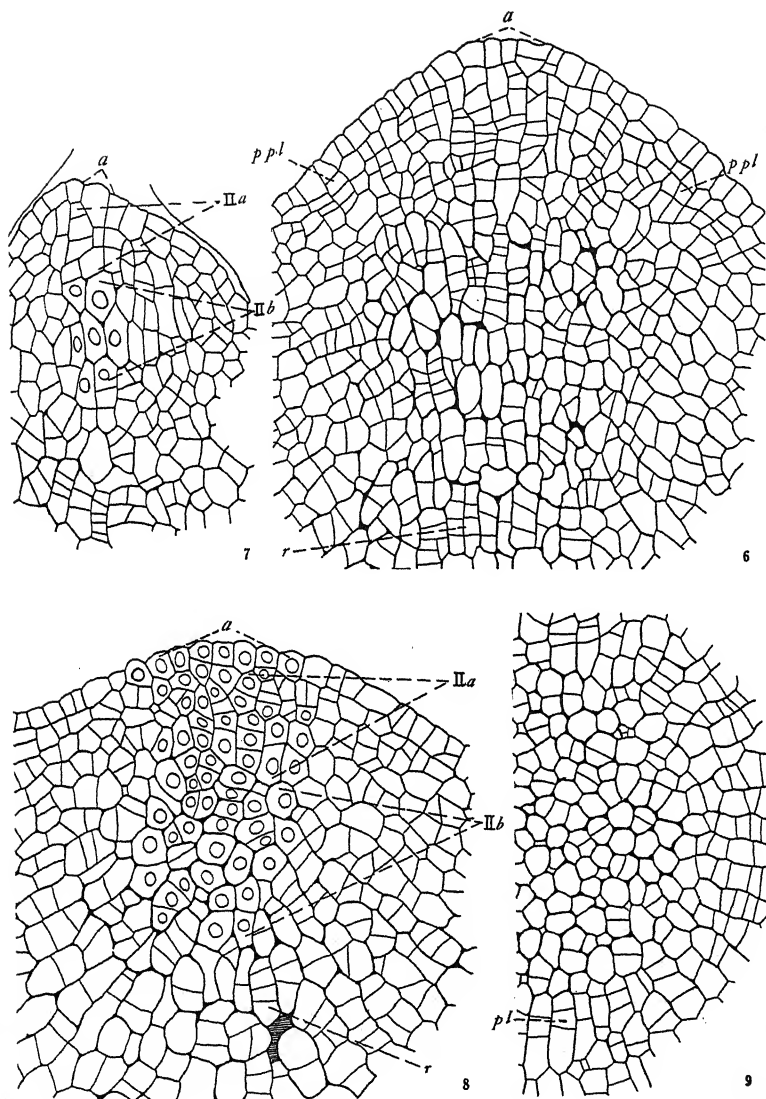
FIGS. 2-5.—Fig. 2, *Z. silvicola*. Median longisection showing cone-shaped meristem. Note vertical alignment in central mother cell zone resulting from periclinal divisions; practically all cells in remainder of meristem radiate from central mother cells. Fig. 3, *Z. umbrosa*. Transection showing cells of apical initial group. Three cells at *a* interpreted as being near geometric center of apex. Note tendency for initials to form tetrads by anticlinal division. Fig. 4, *Z. integrifolia*. Transverse section through cone-shaped apex. Note tetrad of apical initials. Fig. 5, section taken 8  $\mu$  below fig. 4. Note base of apical tetrad in center and irregular arrangement of surrounding initials.

will be recalled that a dolabrate cell cuts off segments alternately by anticlinal division from each of its curved lateral faces, and that in the pyramidal type anticlinal walls carve segments from each of the three lateral triangular faces in sequence. Figures 3 and 4, transverse sections through the apex, show that in *Zamia* the initials may be divided into tetrads by anticlinal walls. Furthermore, when viewed in longitudinal section (fig. 8) it is seen that periclinal divisions occur,

which is not the case in either dolabrate or pyramidal apical cells. It follows, therefore, that the structural affinities of the apical initial complex lie not with the cryptogams as a whole but rather with the scheme shown by FOSTER (4) for *Ginkgo*; by KORODY (10) for *Abies*, *Picea*, and *Pinus*; and by HÄRTEL (5) for a number of species of *Lycopodium*.

#### ZONE II: CENTRAL MOTHER CELLS

This zone is of special interest because, with the exception of *Ginkgo*, it appears to be unique among plants. The general scheme of the cell pattern, as it appears in median longitudinal section, may be obtained from figures 2, 6, 7, and 8. The first impression is that of a central core of vertically aligned cells extending some 200 to 400  $\mu$  into the heart of the meristem, from which the cells of zones III and IV radiate in more or less regular rows. The apical initials contribute to the central mother cells by periclinal divisions. The inner layer of daughter cells behaves much as initials, in that the cells divide both anticlinally and periclinally (the latter divisions predominate), so that tiers of cells from four to twelve layers deep are built up. This region, designated in figure 1 as zone IIa, is apparently one of cell multiplication. Unfortunately direct evidence for the actual rate of division has not been found in any of the material examined. The only mitotic figures observed were in two seedlings from lot IV. In view of the number of apices studied and the variety of fixing agents used, some explanation is necessary to account for the failure in locating mitosis. The plants of *Zamia integrifolia*, which were fixed immediately in the field, bore leaves and cones in many stages of development. Care was used in removing the tissues surrounding the bases of the tips in order to insure rapid fixation. Material from the greenhouse, and a few plants which had been brought into the laboratory one week after collection in the field, were fixed under reduced air pressure to make quick penetration certain. Negative results were obtained in both cases. The interval between collection and fixation was apparently not excessive, for COULTER and CHAMBERLAIN (2) obtained mitosis in ovules from cones which had been in the laboratory for two weeks. Penetration must have been satisfactory, for division figures occurred in seed-



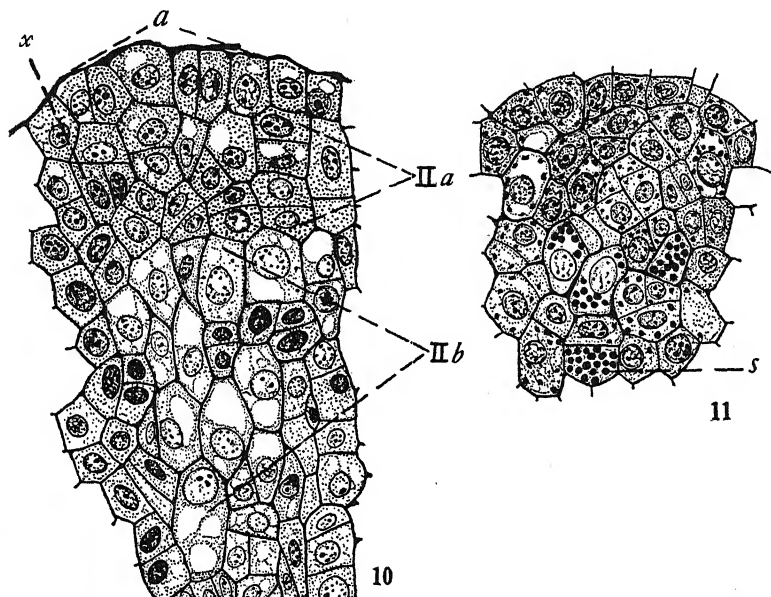
FIGS. 6-9.—Fig. 6, *Z. umbrosa*. Median longisection with gently rounded apex. Note tiers of cells in peripheral layers derived from superficial layer (*ppl*), also extensive development of central mother cell zone with oblique-anticlinal cleavage contributing to zone III. Fig. 7, *Z. integrifolia*. Median longisection through shoot apex of mature embryo. Note enlarged central mother cells, periclinal divisions in superficial layer, and large apical initial which might be mistaken for an apical cell. Drawing made from slide prepared by Dr. M. A. CHRYSLER. Fig. 8, *Z. integrifolia*. Median longisection through mound-shaped apex. Note strong tendency for cells to radiate from central mother cell zone and oblique anticlinal divisions through which they have been derived; also origin of rib meristem from rejuvenated central mother cells; and that cells derived from superficial layer may divide anticlinally. Fig. 9, *Z. integrifolia*. Transection through inner peripheral layers showing radial arrangement and origin from central mother cells.

lings fixed with the same treatment as that used for the mature plants. The discovery that the majority of the apices were gorged with starch suggested that protein synthesis had been retarded sufficiently to throw the meristematic tissues into a period of dormancy so far as mitosis is concerned. This seems likely when it is recalled that the cycads are noted for their exceedingly slow growth and their habit of suddenly producing cones or a crown of leaves following periods during which signs of growth were not visible. Material would have to be collected over a longer period of time than has been available in order to determine the duration of the dormant and active periods. Thin cell walls, large, deeply stained nuclei and dense cytoplasm, in addition to paired cells, have been considered as sufficient proof for indicating recent division.

The actively dividing cells (zone IIa) by either gradual or abrupt transition merge into zone IIb, which contains the most conspicuous cells in the meristem. In median longitudinal sections they are seen to be about twice as long as their peripheral neighbors, and to have thin, highly vacuolated cytoplasm and large, faintly stained nuclei (fig. 10). Deeply stained thickenings often occur where several cells make contact. This feature is not nearly so prominent as has been figured by FOSTER (4) for *Ginkgo*, and may be absent (fig. 10). The enlarged cells are seen less readily in transverse section, owing in part to the fact that all the cells have about the same diameter. Critical examination of cell contents, however, reveals their presence (fig. 11). Division may occur deep within the central mother cell zone, cleavage being anticlinal, periclinal, or oblique (figs. 10, 11). As a result the enlarged cells become isolated among small ones and the conspicuous feature of the zone seems to have disappeared. Thus while zone IIb is generally regarded as a region of enlargement, it might be more accurate to think of enlargement as an ultimate characteristic of the central mother cells before they return to the meristematic condition leading to final differentiation. For the most part the enlargement phase is reached by the majority of the cells at the same time, but exceptions occur, as already noted.

• The central mother cell zone, when considered as a whole, is obviously a region of increase in volume, accomplished by both cell division and cell enlargement. Its most significant characteristic,

regardless of its structure or stage of cellular activity, is that it serves as the point of origin for most of zone III and all of IV. No exception has been found to this statement in the fifty tips examined. On the other hand, nothing approaching this condition can be seen in the excellent figures of conifer apices in the comprehensive work of KOCH (9) or KORODY (10).



FIGS. 10, 11.—Fig. 10, *Z. integrifolia*. Median longisection showing portion of central mother cells in detail. Note anticlinal divisions in apical initials; periclinal and oblique periclinal divisions which build up actively dividing part of central mother cell zone; anticlinal division in central mother cell at *x* contributing to superficial layers; enlarged central mother cells with large faintly stained nuclei and vacuolated cytoplasm; also small cells from recent division within central mother cell zone. Fig. 11, *Z. silvicola*. Transection through enlarged cells of central mother cell zone. Note prominent starch grains (*s*).

### ZONE III: PERIPHERAL LAYERS

The peripheral layers flank the central mother cells and owe their origin to them and the outer layer of the meristem below the apical initials. In apices containing starch this zone can be subdivided into III*a* and III*b*. The lower part (III*b*) is composed of some six rows of cells (fig. 8) radiating from the lower portion of the central mother

cell group. Division is somewhat obliquely periclinal, and the newly formed cells appear to be in filaments revealing their common origin from a single mother cell. New filaments or rows of cells may be started from divisions parallel to the surface which tend to broaden the peripheral layers and give them a fanlike appearance. Transverse sections taken at this level (III*b*) supply convincing evidence that the radiating rows of cells have their origin in a transition zone belonging to the central mother cell group (fig. 9).

The outer peripheral layers, generally about four in number, are derived from two sources. Some of the cells result from anticlinal divisions in the upper four or five layers in the central mother cell zone which have not passed through the enlargement phase characteristic of the members deeper within that zone; the others have been derived from periclinal divisions in the surface cells. The daughter cells from the latter source divide periclinally, and build up tiers of cells three and four layers in depth (figs. 2, 6, 8). *Zamia* differs from *Ginkgo* in that a much greater contribution is made to the peripheral layers by these cells.

Continued development in the peripheral layers results in formation of leaf primordia, cortex, vascular tissue, and probably the outer part of the pith, in the order named.

#### ZONE IV: RIB MERISTEM

SCHÜEPP (12) has introduced the term rib meristem to indicate a primary meristem concerned with growth in length. The cells are arranged in vertical rows which have been produced by continued transverse division of the mother initials. The term is used here even though elongation in the stem of the Florida species of *Zamia* is much reduced.

The central mother cells lie directly above the pith and contribute to it through the medium of a shallow rib meristem (figs. 2, 6, 8). The divisions are periclinal and form short filaments, but vertical cleavage also occurs and thereby increases the diameter of the meristem. The presence of a rib meristem indicates convincingly that the enlarged central mother cells represent a phase in the growth of the meristem proper, for without it they would be regarded as maturing pith cells.

## OCCURRENCE OF STARCH

The presence of starch is not unexpected in the tissues of a plant which has the reputation of being one of the few North American species exploited commercially for this product. It is surprising, however, to find that the meristem which is normally concerned with protein synthesis should become gorged with stored carbohydrate. Thin sections through the shoot apices from lot III, when treated with iodine, gave a positive reaction for starch in all four zones, although it was most pronounced in zones III and IV. In some cases the entire superficial layer gave a strong positive test, while in others only a few cells in the central mother cell zone contained starch. In general, zones I, II, and IIIa were most likely to be starch free. This made them stand out and assume an outline not unlike that of a circular topped glass stopper inserted into the broad cone-shaped apex.

The storage of starch throws light on certain problems in meristem function as a whole. First, it shows that a meristem may be shifted from protein to carbohydrate synthesis with a period of dormancy as a result. Second, it shows that large amounts of soluble food must readily penetrate the meristem, which would be of great importance where the meristem is a massive structure as in *Zamia*. If the cell wall serves in this capacity, as has been proposed by PRIESTLEY and TUPPER-CAREY (11), it must offer an efficient pathway for translocation even though the time for penetration may be long. Third, it shows that the conversion of the starch reserves to soluble form provides food for rapid growth in all parts of the meristem, and thus reduces the necessity of translocation over long distances, however it may occur.

## Discussion

Fundamentally the architecture of the shoot apex is the same in the Florida species of *Zamia* as in *Ginkgo biloba*. Each has a meristem composed of four distinct zones, the combination of which is not found elsewhere among plants. The apex is more massive in *Zamia* than in *Ginkgo* and it continues to increase in size as the plant ages and the maximum number of leaves per whorl is reached. The largest specimen examined had a cone with partly matured seeds, a crown of fourteen well developed leaves, and an apical meristem

measuring  $856\ \mu$  in diameter  $156\ \mu$  from the apex. A comparison zone by zone shows that the apical initials are more numerous in *Zamia*; that the central mother cells are more active near the apex and are more inclined to divide periclinally than in *Ginkgo*, with the result that they accumulate in vertical rows as seen in median longitudinal sections. This subzone becomes a well marked feature in *Zamia*, but is represented in *Ginkgo* by the subapical layer of cells which in part may function as initials by dividing periclinally. In *Zamia* periclinal divisions are more common than anticlinal. The latter do occur, however, especially at the periphery of the region. The lower central mother cells are more inclined to be in vertical rows, and are more often isolated by small cells than in *Ginkgo*. The outer layers of the peripheral meristem have their cells arranged more nearly in vertical tiers than has *Ginkgo*, which suggests that they have been derived largely from periclinal divisions in the surface layers. The rib meristem is shallow and compares favorably with that in the spur shoots of *Ginkgo*.

FOSTER (4), comparing the shoot apex of *Ginkgo* with that of other gymnosperms, concludes that the closest similarity lies in the Abietaceae, which have an apical cell group resembling that of *Ginkgo* in form and behavior, and furthermore lack a typical "dermatogen." Comparison between *Zamia* and the Abietaceae warrants the same conclusion, but it must be borne in mind that the studies on the Abietaceae by KOCH (9) and KOROVY (10) fail to show the presence of a zone comparable in any respect with the central mother cells from which the remainder of the meristem radiates in *Ginkgo* and *Zamia*.

The more nearly vertical arrangement of cells in the central mother cell zone might be argued as placing *Zamia* nearer to the condition attained in the Abietaceae. This similarity is not advanced as illustrating relationship, since the unique characteristic of a central region serving as "cambium" for the remainder of the meristem is not approached in the conifers.

No attempt, even if feasible, can be made at this time to determine the degree of evolutionary relationship between *Ginkgo* and the cycads based on the organization of the shoot apex. STRASBURGER (13) figures (table 25, fig. 36) a longitudinal section through the stem tip of *Cycas revoluta* in which the existence of a central zone somewhat

comparable with the central mother cell zone in *Zamia* is suggested but by no means proved.

It would follow, should the condition as seen in *Zamia* be typical, that the occurrence of such similarity of structure in forms as widely separated as the cycads, which were derived from the Cycadofilicales, and *Ginkgo* with its ancestry in the Cordaitales, will doubtless be of significance in an understanding of the evolutionary development in the shoot apex of gymnosperms.<sup>2</sup>

### Summary

1. The shoot meristem of *Zamia integrifolia*, *Z. silvicola*, and *Z. umbrosa* consists of four distinct zones, as follows:

Zone I, a group of superficial initials occupying the broad apex of the shoot. The entire meristem has its origin in these cells.

Zone II, a central core of mother cells derived by periclinal divisions from zone I. This is a region of increase in volume accomplished by rapid cell division in its upper layers and by cell enlargement in the lower. These later are rejuvenated and contribute to the surrounding meristematic zones. *Ginkgo* seems to be the only other vascular plant so far investigated which duplicates this region.

Zone III, a series of peripheral layers flanking zone II. The outer layers originate from the superficial cells of the apex and the inner ones by oblique divisions of the central mother cells. Leaves, cortex, vascular tissue, and probably a portion of the pith originate in this zone.

Zone IV, a shallow rib meristem of vertically aligned cells, produced entirely from the base of the central mother cell zone and separating it from the maturing pith.

<sup>2</sup> The recent work of FOSTER (FOSTER, A. S., Structure and growth of the shoot apex of *Cycas revoluta*. Amer. Jour. Bot. 26:372-385. 1939) has not been discussed since it was not received until after the present paper had been submitted for publication. A close similarity exists in the structure and zonation of the shoot apex in *Cycas* and *Zamia*. My interpretation in *Zamia* has been based on the general plan seen in *Ginkgo* but with sufficient modifications to bring it in line with the interpretation given by FOSTER for *Cycas*. The principal difference seems to be in the growth of the peripheral layers; in *Cycas* the cell pattern is highly variable, as is also the case for young *Zamia* plants. In large specimens with moundlike apices the cell pattern is still variable, however, but there is a well marked tendency for the cells to be stratified in almost vertical rows. These originate from periclinal (to the apex) divisions of the superficial layer as well as of the layers beneath.

2. The occurrence of starch throughout the shoot apices in plants collected during January and March suggests that the meristem undergoes alternating periods of dormancy and active growth.

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# HISTOLOGICAL AND PHYSIOLOGICAL RESPONSES OF BEAN PLANTS TO ALPHA NAPHTHALENE ACETAMIDE<sup>1</sup>

E. J. KRAUS AND J. W. MITCHELL<sup>2</sup>

(WITH FIFTEEN FIGURES)

## Introduction

During the past several years, thirty-six growth promoting substances have been tested to determine their effect on the histological development of plants to which they were applied, and also to test some of their physiological effects. Some of these compounds have been listed by TRAUB (10) with respect to their effect in promoting root development when applied to stem cuttings. Among those tested, one, alpha naphthalene acetamide, m.p. 184°, produced by the American Chemical Paint Company, resulted in such striking and characteristic effects that it has seemed worthwhile to invite attention to the results of several experiments in which it has been used.

This substance is but sparingly soluble in either water or lanolin. It was employed in the following ways: (1) it was applied to the cut surfaces of bean, African marigold, and *Mirabilis* stems as a 2 per cent mixture in anhydrous lanolin; (2) it was sprayed on to the surfaces of these and other species as emulsions of varying concentration; and (3) cuttings of various plants were partially immersed for varying lengths of time in aqueous solutions of it.

When the 2 per cent lanolin mixture was applied to the cut surfaces of young, 3 cm. long, decapitated second internodes of the bean or to first internodes of *Mirabilis*, the gross responses were strikingly different from those of all other substances tested. Following application there was some further elongation of the internodes, very little proliferation of tissues to form an apical tumor, and a few roots

<sup>1</sup> This investigation was aided in part by a grant to the University of Chicago from the Rockefeller Foundation.

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emerged near the surface of application, especially if the treated plants were subsequently placed in a saturated atmosphere (5). Other noticeable features were the greatly increased secondary thickening of the entire stem below the surface of application, especially the differentiation of secondary xylem, the decided thickening of the walls of the cells of most of the tissues composing the stem, and the partial suppression of elongation of axillary buds.

The most obvious characteristic was the greatly increased firmness or hardness of the treated internodes and in the case of *Mirabilis* the failure of the treated internodes to absciss even after many weeks, although ordinarily they will do so in eight or ten days when treated with lanolin only or if decapitated and left untreated. A delay of abscission has already been noted as one of the effects of application of lanolin indoleacetic acid mixtures to *Mirabilis* (2), but the effect is much less pronounced or lasting than when alpha naphthalene acetamide is used. In the case of the latter, secondary thickening takes place so quickly and so extensively after its application either in lanolin mixture or as a spray, and these effects are so extensively distributed throughout the plant (fig. 1), that its use may well prove of economic importance when it is desired to increase the degree or rate of secondary thickening or in the prevention or delay of abscission of plant organs such as leaves, flowers, fruits, etc. Its effect on parthenocarp in holly has been demonstrated by GARDNER and MARTH (1).

In the illustrations which are used in this paper, the specific experimental treatment imposed is given in the legend for each figure and the histological details are generally obvious. As already stated, one of the most evident features is the effect of the treatments on the degree and rate of increase of the xylem and on wall thickening. The root systems of treated as compared with untreated plants are much more fibrous. When roots are produced by the stems following application of a 2 per cent lanolin paste, their origin is generally from the derivatives of the cells of the rays (figs. 2, 4). Frequently the endodermal cells proliferate and from them vascular bundles are differentiated (fig. 5). The cells of the pith in the vicinity of the vessels also may divide (fig. 8), and from these vascular strands may be differentiated. The latter effects are similar to those resulting from ap-

plications of indoleacetic acid lanolin mixtures (3, 5) but are much less pronounced within the same period of time after treatment. Because of its relatively low solubility, the effective concentration of alpha naphthalene acetamide which may penetrate to or reach cells at some distance from the point of application may also be low. But it is clearly obvious that even though the concentration is low there is a ready penetration of the tissues for appreciable distances from the surface of application. This is indicated in the responses shown by entire plants whose leaves have been sprayed with emulsions of it (fig. 1) and in the increased activity of the cambium, the amount of secondary xylem differentiated, and the extensive thickening of the cell walls (fig. 7).

In addition to the numerous experiments in which applications were made to stems and other parts of various species of plants to study the subsequent histological development, several physiological experiments were conducted to determine: (1) the effect of alpha naphthalene acetamide on bud inhibition; (2) its effect on the accumulation of solid substances in the part of the plant to which the chemical was applied; and (3) the histological responses as compared with those resulting from indoleacetic acid. Since the results of comparable experiments have been similar, details of only two are presented here.

### Investigation

#### EXPERIMENT I

Kidney bean plants were grown in soil in the greenhouse until the second internodes were 2.5-5 cm. long and the first trifoliate leaf was beginning to expand and flatten out. All the plants were then decapitated by severing the second internodes about 1.5 cm. above the node at which the heart-shaped leaves are borne. They were then divided into four equal lots. To the cut surfaces of one lot a thin smear of a 2 per cent mixture of lanolin-alpha naphthalene acetamide was applied, to another a 2 per cent mixture of lanolin-indoleacetic acid, to a third lanolin only, and the fourth was left without further treatment. The several treatments were given at random over two large benches, and involved several hundred plants.

One week later the plants were harvested and divided into the fol-

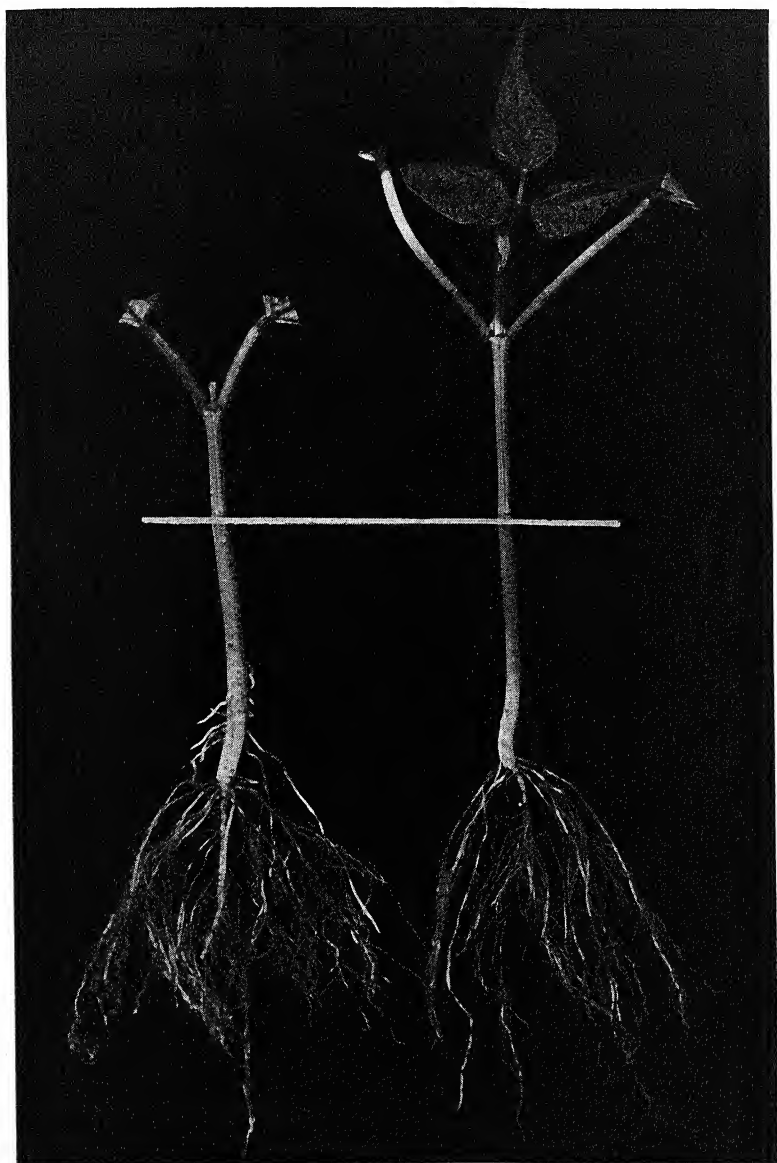


FIG. 1.—Plant (at left) 5 days after primary leaves had been sprayed with emulsion of alpha naphthalene acetamide, 625 mg. per liter; plant at right sprayed with lanolin emulsion only. Primary leaves 3–4 cm. long; second internode not yet elongated at time of spraying. Blades of primary leaves removed when photographed. The white line passes through the cotyledonary node. The lesser elongation and increased thickness of all above-ground parts (except second internode); the finer, more fibrous root system; and the initiation of roots on the stem below the primary leaves are characteristic effects following spraying with this growth substance.

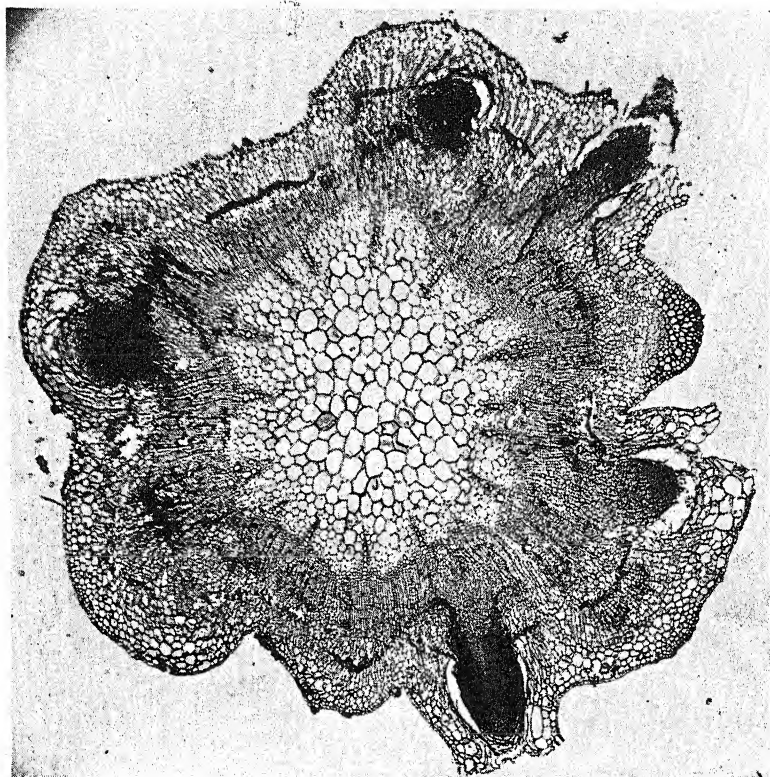


FIG. 2.—Transverse section (about 1.5 mm. below treated surface) of second internode of decapitated bean plant 20 days after treatment of cut surface with 2 per cent alpha naphthalene acetamide-lanolin mixture. Plants maintained for last 5 days of period in humid atmosphere. Relation of roots to rays, a few vascular strands from endodermal derivatives, and great increase in secondary xylem are characteristic. Cf. fig. 3.

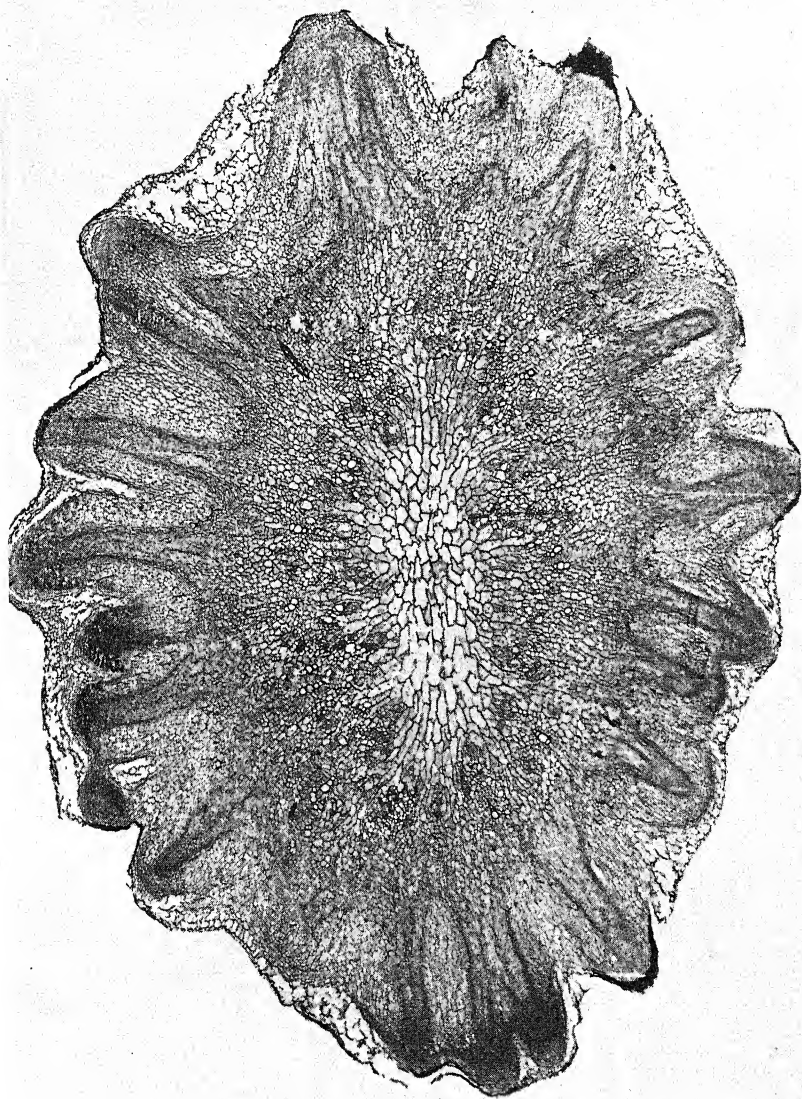


FIG. 3.—Transverse section (about 1.5 mm. below treated surface) of second internode of bean plant 7 days after treating cut surface with 3 per cent indoleacetic acid-lanolin mixture. Compared with section shown in fig. 2, the much greater development of roots and the greater proliferation of pith, phloem, and other tissues are characteristic.

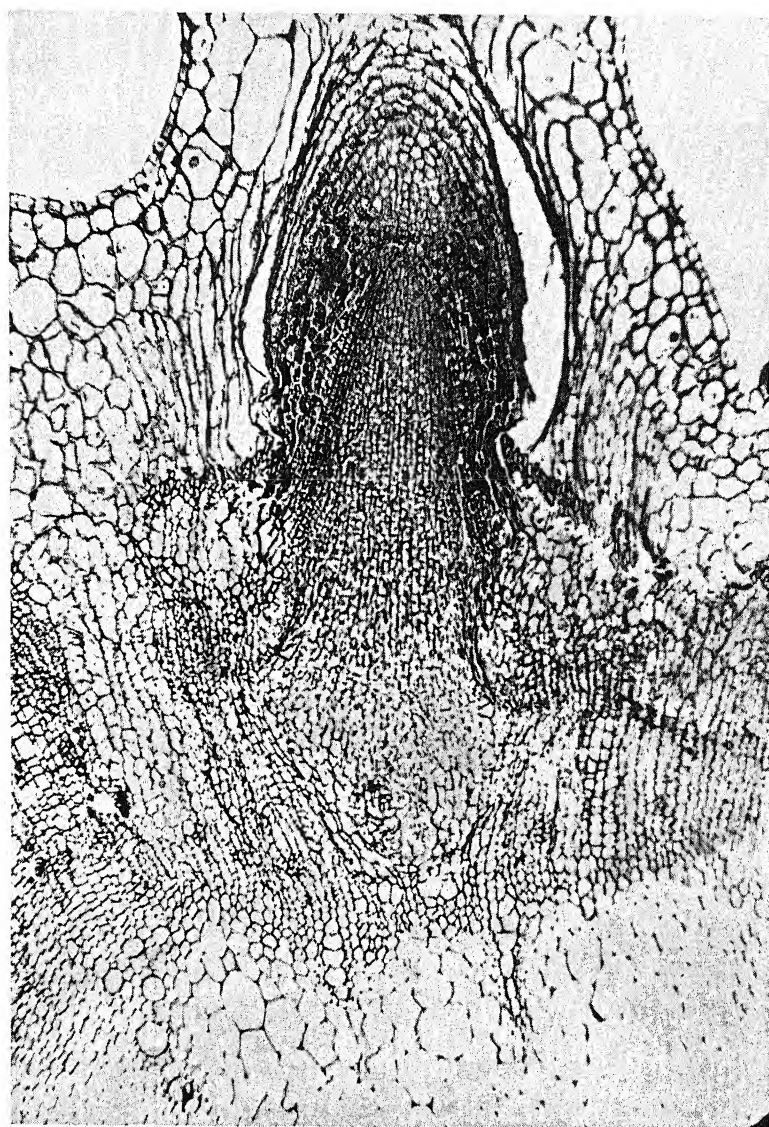


FIG. 4.—Enlarged view of young root shown in fig. 2. Pericyclic fibers are thick walled and disrupted by the root having passed through them.

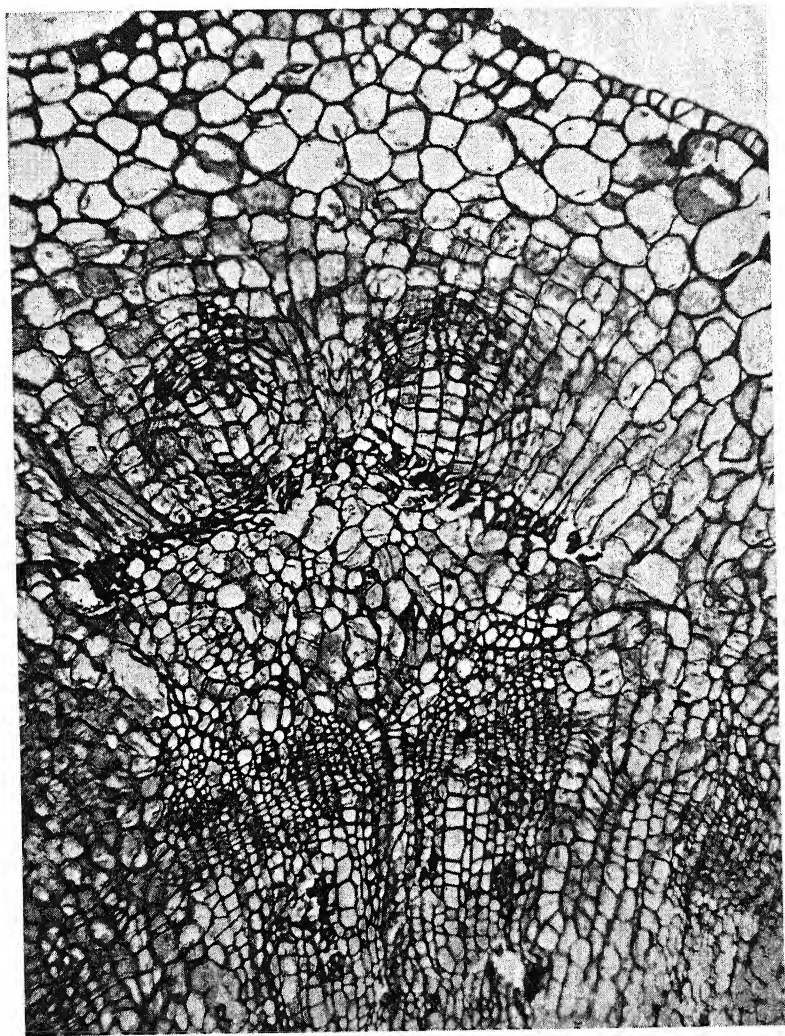


FIG. 5.—Portion of section shown in fig. 2 greatly enlarged. Two vascular bundles differentiating from derivatives of endodermis. Pericyclic fibers much thickened; primary phloem only slightly meristematic.

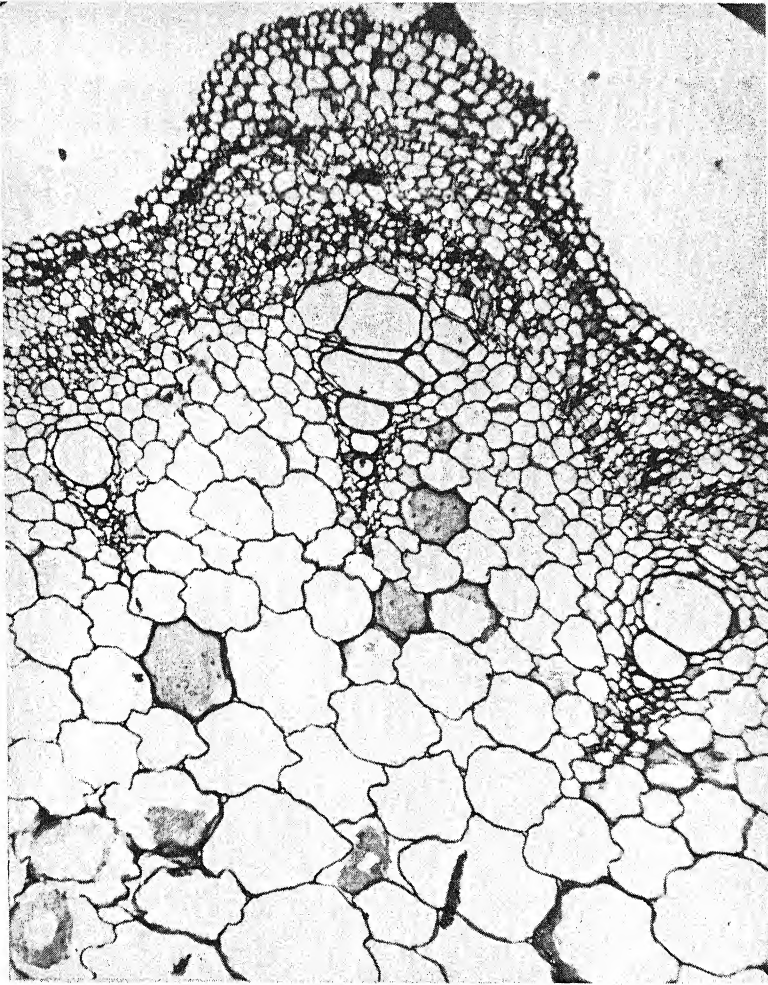


FIG. 6.—Section of second internode decapitated when trifoliate leaf had just flattened and treated surface smeared with lanolin. Section 15 mm. from treated surface, 20 days after treatment. There has been relatively little increase in secondary xylem, although there has been a partial maturation of most other tissues. Cf. fig. 7.

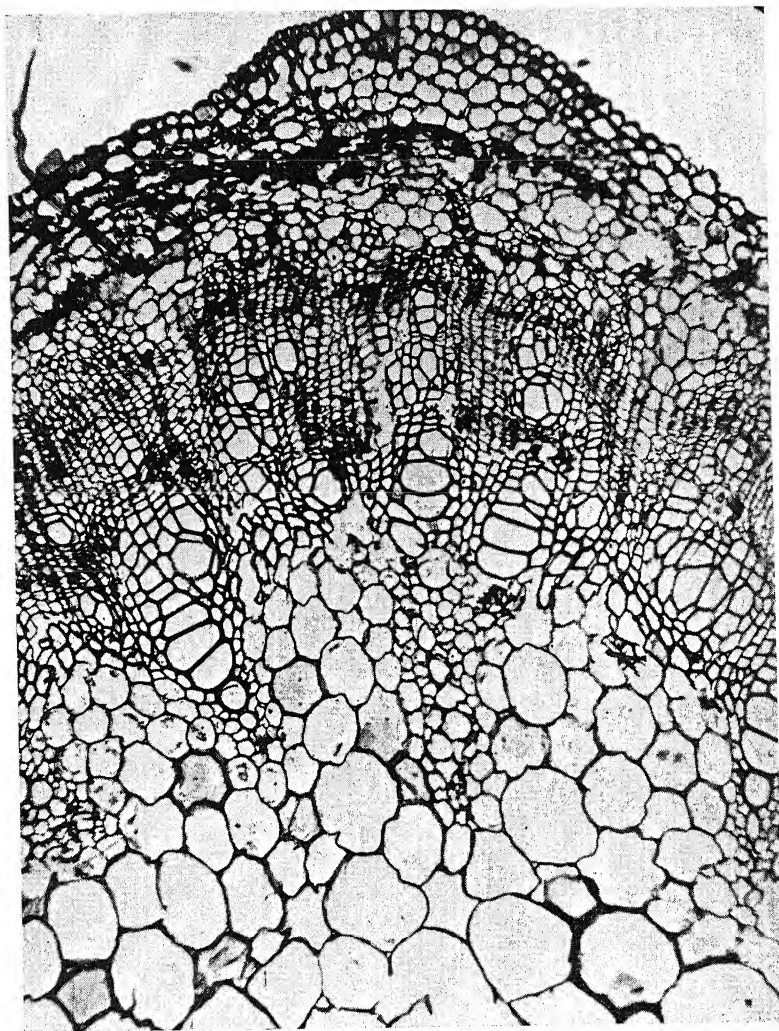


FIG. 7.—Internode of same age as one shown in fig. 6, cut surface treated with 2 per cent alpha naphthalene acetamide-lanolin mixture. Section 15 mm. from treated surface, 20 days after treatment. Relatively great development of secondary xylem and increased thickening of walls of cells of other tissues are characteristic.

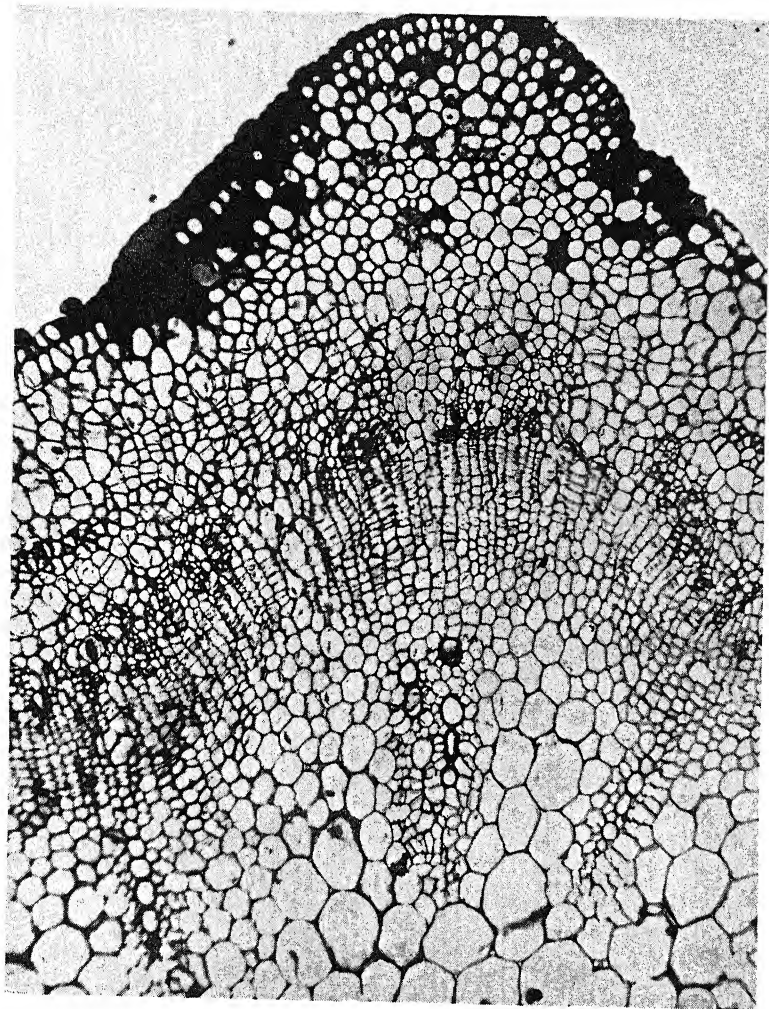


FIG. 8.—Same internode as shown in fig. 7 except that the section is just immediately below the treated surface. There is some proliferation of pith cells adjacent to xylem vessels, of phloem and cortical cells, and some cells have died. Immediately below this level there is increased secondary thickening for long distances down the treated internode and throughout other portions of stem below it. *Cf.* fig. 7.

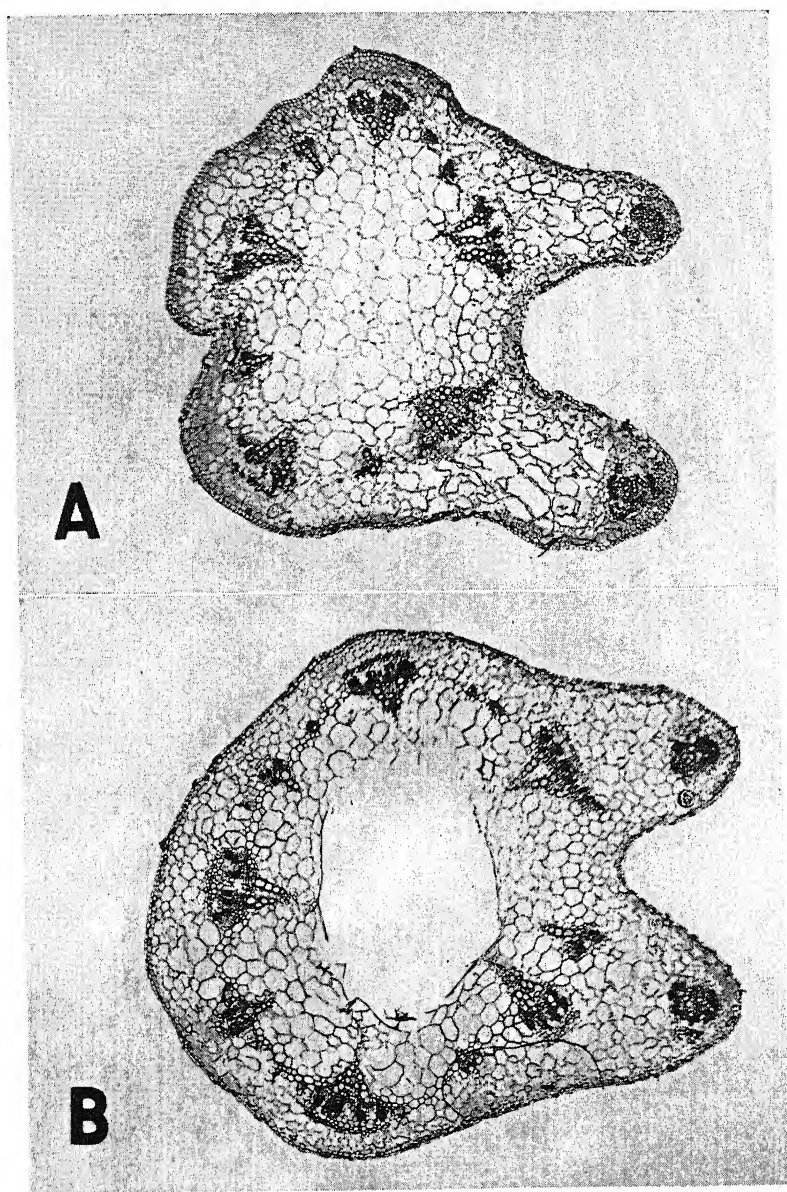


FIG. 9.—Petioles from bean plants used in experiment II. *A*, sprayed with emulsion containing lanolin only; *B*, with alpha naphthalene acetamide-lanolin emulsion; both harvested 8 days after first application of spray. Greater diameter of petiole, greater amount of secondary xylem, increased thickness of cell walls, and tearing of parenchyma at center (as shown in *B*) are characteristic. Cf. figs. 10 and 11.

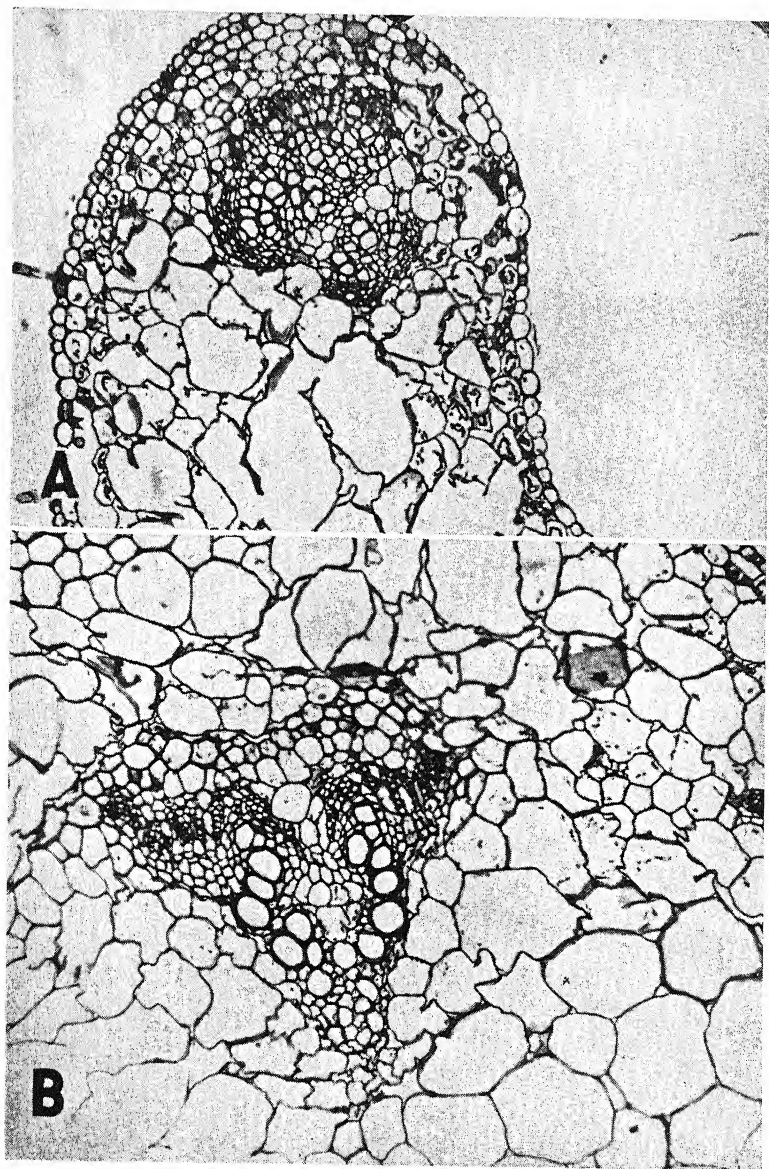


FIG. 10.—Portions of petiole shown in fig. 9A greatly enlarged. A, vascular bundle in ridge of petiole; B, one of larger bundles just above middle at left in fig. 9A.

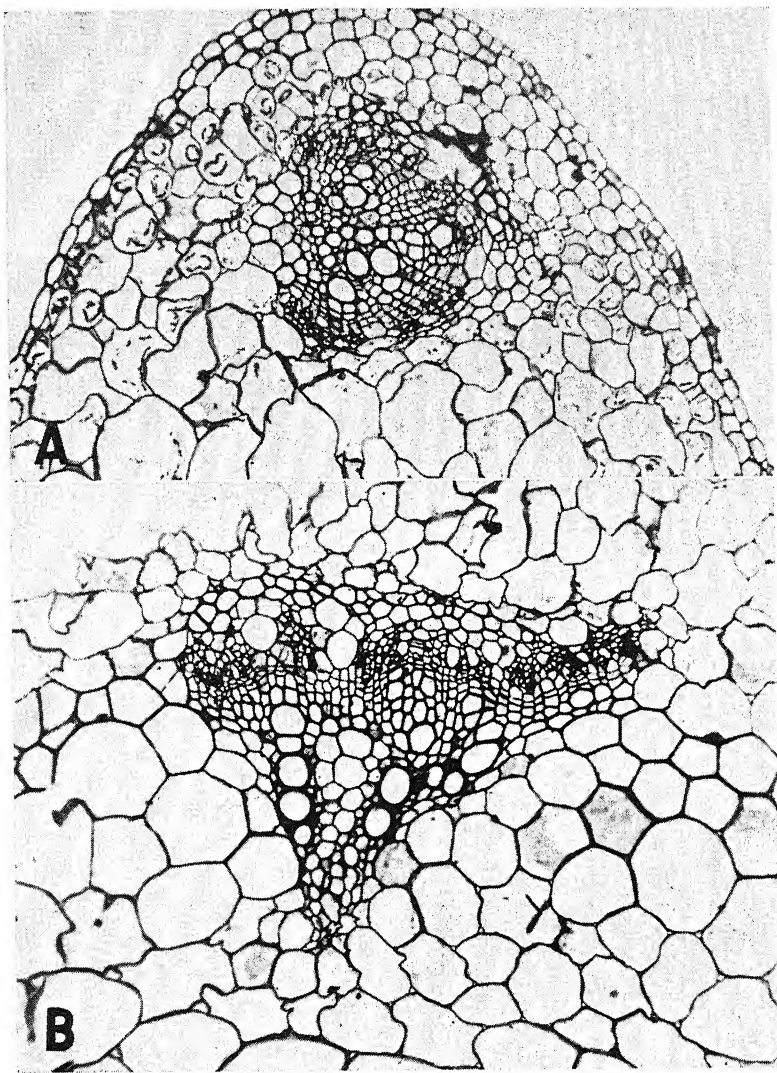


FIG. 11.—Portions of petiole shown in fig. 9*B* greatly enlarged. *A*, *B*, bundles in same relative position as described in fig. 10. Greater thickness of walls of parenchymatous cells and increased secondary xylem are characteristic.

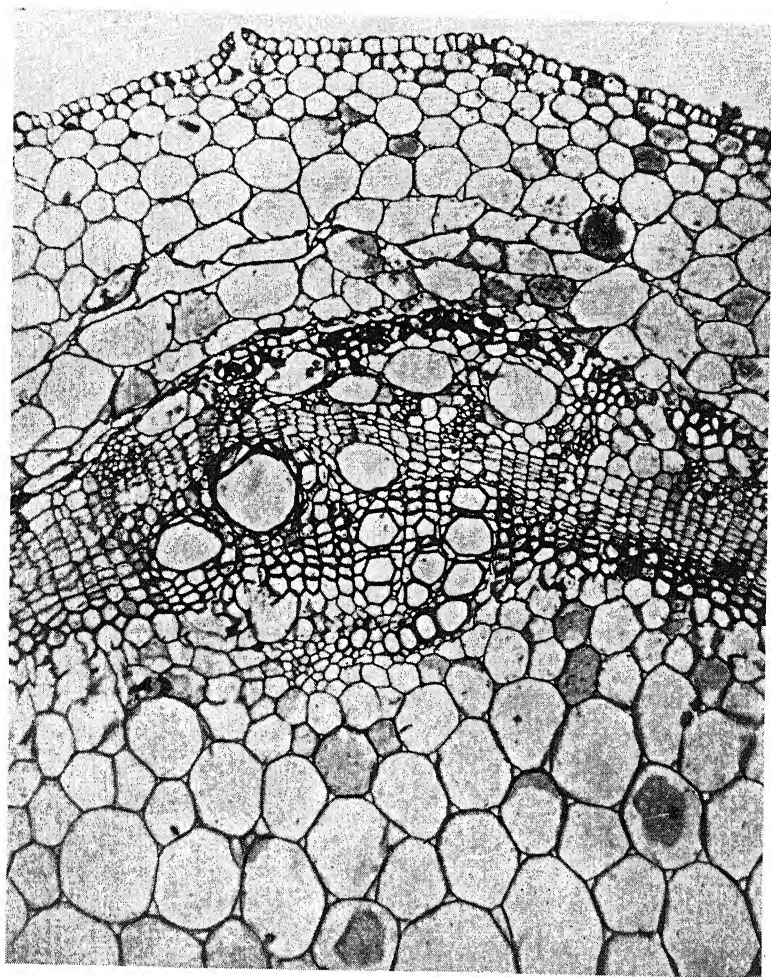


FIG. 12.—Transverse section of mid-region of hypocotyl of plant of experiment II 8 days after first spraying primary leaves with lanolin emulsion.

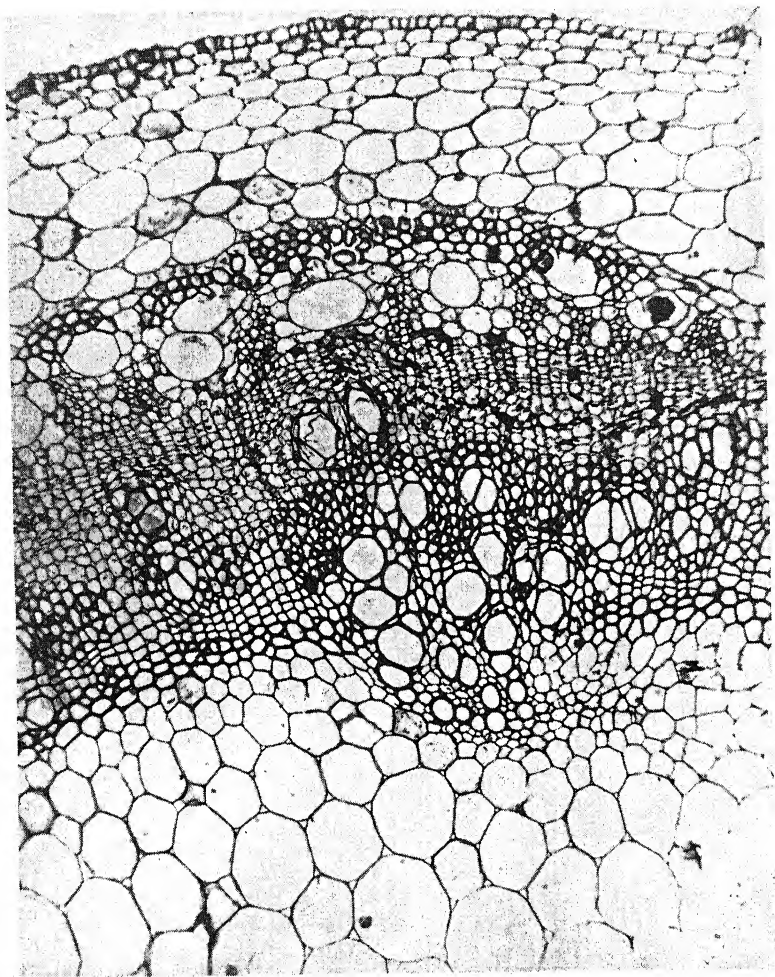


FIG. 13.—Transverse section of mid-region of hypocotyl of plant of experiment II 8 days after first spraying primary leaves with emulsion of lanolin-alpha naphthalene acetamide. Cf. hypocotyl shown in fig. 12; amount of secondary xylem is very much greater as are also the wall thickenings of several other tissues.

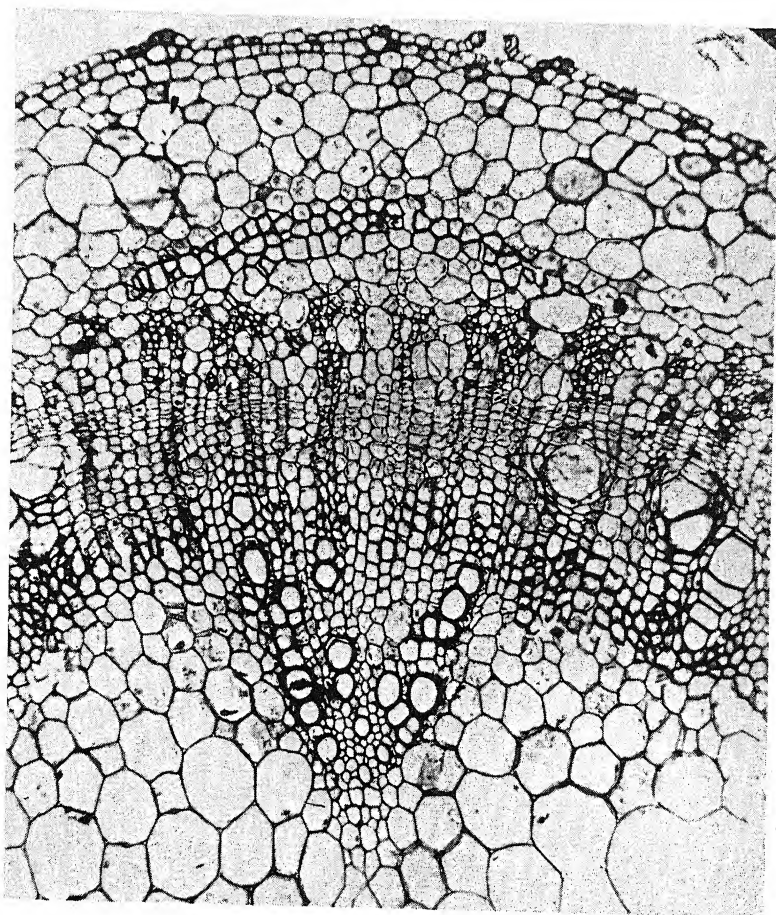


FIG. 14.—Transverse section of mid-region of first internode of plant of experiment II 8 days after first spraying primary leaves with lanolin emulsion.

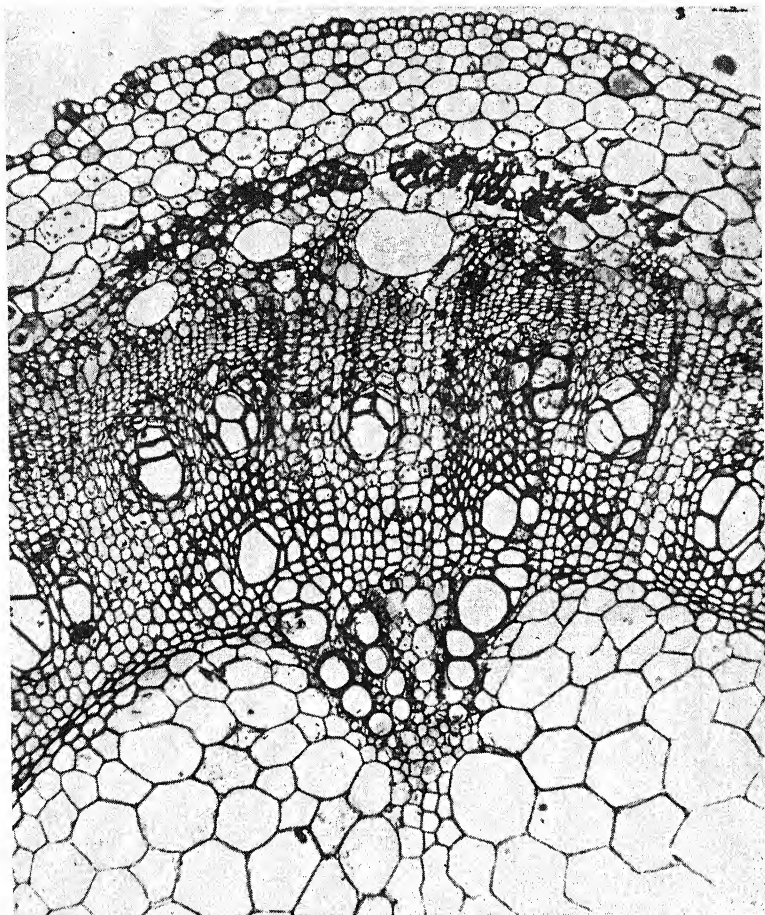


FIG. 15.—Transverse section of mid-region of first internode of plant of experiment II 8 days after first spraying primary leaves with lanolin-alpha naphthalene acetamide emulsion. Relatively greater development of secondary xylem and wall thickening as compared with plant sprayed with lanolin emulsion only is even more pronounced than in the hypocotyl.

lowing fractions: first internode, second internode, primary leaves, and axillary shoots. The fresh weight of corresponding parts from 200 plants of each treatment was recorded and the dry weights determined after drying in a well ventilated oven at 80° C. (table 1).

It is evident from the data of table 1, as well as from the histological evidence, that alpha naphthalene acetamide resulted in the mobilization of solid substances toward the place of application, but that this effect is of less magnitude than that which resulted when

TABLE 1

WET AND DRY WEIGHTS OF PARTS OF PLANTS TREATED WITH 2 PER CENT PASTES OF LANOLIN AND ALPHA NAPHTHALENE ACETAMIDE, LANOLIN AND INDOLEACETIC ACID, AND PURE LANOLIN, AS COMPARED WITH WEIGHT OF CORRESPONDING PARTS OF UNTREATED PLANTS. FIGURES REPRESENT GRAMS WEIGHT OF PARTS FROM 200 PLANTS

TREATMENT	AXILLARY SHOOTS		SECOND INTERNODE		FIRST INTERNODE		PRIMARY LEAVES
	WET WEIGHT	DRY WEIGHT	WET WEIGHT	DRY WEIGHT	WET WEIGHT	DRY WEIGHT	WET WEIGHT
Untreated.....	535.5	58.1	17.0	3.7	69.0	15.0	572.0
Lanolin.....	525.0	57.0	19.0	4.2	70.0	15.6	569.0
Lanolin and alpha naphthalene acetamide.....	372.0	41.2	31.0	8.1	82.0	20.0	650.0
Lanolin and indoleacetic acid.....	328.5	32.4	53.8	13.1	82.8	20.0	607.0

indoleacetic acid was applied in the same percentage composition in lanolin mixtures. As already suggested, a direct comparison of the magnitude of the responses resulting from the use of naphthalene acetamide and those resulting from the use of indoleacetic acid cannot be made unless the possible differences in the relative solubilities of the two substances in lanolin or the liquids in the plant, or in their capacity to move through the plant, is taken into account. Various experiments designed to determine some of these points will be reported in the future.

#### EXPERIMENT II

Several hundred bean seeds were planted in rows in soil on a greenhouse bench. At the end of the following week the plants were approximately 8 cm. above the soil, the primary leaves averaged 4 cm.

in length, but the second internodes had not yet elongated. Plants in the first row were sprayed with a lanolin emulsion (4) containing 300 mg. of alpha naphthalene acetamide per liter; those in the second row with lanolin emulsion without the acetamide; those of the third row were left untreated. These treatments were replicated twelve times in the same order so that the various treatments occurred evenly throughout the entire length of the bench.

TABLE 2

FRESH AND DRY WEIGHTS AND LENGTH OF DIFFERENT PARTS OF BEAN PLANTS SPRAYED IN SEEDLING STAGE WITH LANOLIN EMULSION CONTAINING 300 MG. ALPHA NAPHTHALENE ACETAMIDE PER LITER AND GROWN FOR 2 WEEKS, AS COMPARED WITH WEIGHTS AND LENGTH OF SIMILAR PARTS OF CONTROL PLANTS OF SAME AGE. WEIGHTS EXPRESSED AS GRAMS PER FIFTY PLANTS

PLANT PART	SPRAYED WITH ALPHA NAPHTHALENE ACET- AMIDE-LANOLIN EMULSION			SPRAYED WITH LANOLIN EMULSION			CONTROL PLANTS		
	WET WEIGHT	DRY WEIGHT	LENGTH (CM.)	WET WEIGHT	DRY WEIGHT	LENGTH (CM.)	WET WEIGHT	DRY WEIGHT	LENGTH (CM.)
Hypocotyl.....	36.7	4.34	5.0	46.8	6.70	5.0	45.6	6.62	5.0
First internode....	26.6	3.25	2.5	37.9	5.04	5.0	37.5	5.08	5.0
Primary leaf petiole	35.0	2.37	4.5	36.8	2.43	7.5	37.0	2.53	7.5
Primary leaf blade.	240.0	20.0	.....	234.0	21.0	.....	239.0	22.5	.....
Tip.....	420.0	40.0	.....	1103.0	117.5	.....	1087.0	115.0	.....
Total.....	758.3	70.0	33.5*	1458.5	152.7	63.0*	1446.1	151.7	63.0*

\* Total height of plants above soil level.

Within 24 hours after spraying there was a noticeable difference in the size of the treated and untreated plants. The second internodes, petioles, and blades of the primary leaves of those treated with alpha naphthalene acetamide showed no appreciable increase in length, while corresponding parts of the control plants or those sprayed with emulsion alone showed a definite increase. On the tenth, and again on the twelfth day after planting, the several treatments were repeated on the same plants using fresh emulsions. During this interval the elongation of those sprayed with alpha naphthalene acetamide was less than that of controls; but the above-ground parts, except the second internode, were of greater diameter and were much

stiffer and firmer to the touch. The plants closely resembled those shown in figure 1, but were slightly larger.

Two weeks after the first spraying, the stems of the plants were cut at the soil level and the above-ground portions divided into five parts: hypocotyl, first internode, petiole of primary leaves, blade of primary leaves, and tips (all parts above the second node). The fresh and dry weights of these parts were determined. The height and total leaf area of treated plants were appreciably less than those of untreated plants. The primary leaves of treated plants were much smaller than those of controls, but the amount of solid matter accumulated in the petioles and blades of these leaves during the experiment was approximately the same. Table 2 shows that plants which were treated with alpha naphthalene acetamide accumulated less solid matter in all the parts measured, and the histological evidence (figs. 9-15) shows that this material was distributed in a very different manner in the two cases, much of it apparently being represented in wall thickenings and as secondary xylem.

Additional experiments are being conducted to determine more precisely what substances are mobilized (and to what extent) within plants treated in various ways with alpha naphthalene acetamide, as has already been done with indoleacetic acid (6, 7, 8, 9). The histological changes and physiological reactions following the application of either are in a few respects similar, but in many other ways these responses are widely different, so that through the use of these compounds the opportunity to study the differential response and development of cells and tissues in relation to growth substances of known chemical composition is appreciably increased.

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## EFFECTIVENESS OF SEVERAL GROWTH SUBSTANCES ON PARTHENO-CARPY IN HOLLY

F. E. GARDNER AND PAUL C. MARTH

An earlier paper (3) gave the results of experiments on inducing parthenocarp in American holly, *Ilex opaca*, by spraying the open blossoms with dilute aqueous solutions of growth substances. Data were presented as to the relative potency of four compounds, indoleacetic, indolepropionic, indolebutyric, and naphthaleneacetic acids, the last named being very much more effective than any of the other three. Recently several other substances have been similarly tried on a comparative basis, and the results, of seeming interest because of their variance in some cases from other reported evaluations of relative effectiveness in plant response, are presented here with the hope that they may eventually help in piecing together the picture of how and why the hormone-like substances bring about the numerous and varied responses associated with their application.

The methods used were essentially those previously reported. The growth substances<sup>1</sup> were dissolved in distilled water and sprayed on the open flowers by means of an atomizer. The tests were applied to small potted plants flowering in the greenhouse and to flowering branches on trees growing on the grounds of the United States Horticultural Station, Beltsville, Maryland.

In table 1 are presented some preliminary results with several compounds of naphthalene on young holly plants flowering in the greenhouse. These plants were found to vary considerably in their ability to set parthenocarpic fruit, depending on their vigor, nutrition, and previous treatment. While only plants selected for their uniformity were used in these tests, yet since 100 per cent setting of fruit was not obtained with treatments which in previous trials had given complete setting, the comparisons were repeated under more uniform conditions. Accordingly the data in table 2 show repetition

<sup>1</sup> Phenylacetic acid was secured from Merck & Company; sodium naphthol(4)-sulphonate from the Eastman Kodak Company; all other naphthalene derivatives from the American Chemical Paint Company, Ambler, Pennsylvania.

of these same compounds and include several others. For these data most of the treatments were applied on branches of a single large

TABLE 1  
PERCENTAGE FRUIT SET INDUCED BY SPRAYING HOLLY  
BLOSSOMS IN THE GREENHOUSE

GROWTH SUBSTANCE	PERCENTAGE CONCENTRATION					
	0.01		0.005		0.001	
	No. OF FLOWERS	PER-CENT-AGE SET	No. OF FLOWERS	PER-CENT-AGE SET	No. OF FLOWERS	PER-CENT-AGE SET
Naphthaleneacetic acid.....	143	89.0	80	46.6	87	3.5
K-naphthalene acetate.....	80	12.5	88	12.5	78	0
Naphthalene acetamide.....	92	98.9	62	90.2	65	36.9
Naphthalene propionic acid*.....			52	38.6	55	0

\* Solubility approximately 0.003 at room temperature.

TABLE 2  
PERCENTAGE FRUIT SET INDUCED BY SPRAYING HOLLY  
BLOSSOMS ON OUTDOOR TREES

GROWTH SUBSTANCE	PERCENTAGE CONCENTRATION					
	0.01		0.005		0.001	
	No. OF FLOWERS	PER-CENT-AGE SET	No. OF FLOWERS	PER-CENT-AGE SET	No. OF FLOWERS	PER-CENT-AGE SET
Naphthaleneacetic acid.....	213	85.0	165	64.8	176	17.0
K-naphthalene acetate.....	308	16.2	179	0	181	0
Naphthalene acetamide.....	294	89.4	121	87.6	182	21.4
Naphthalene thioacetamide.....	191	9.4	173	10.4	145	0
Phenylacetic acid.....	226	0	177	0	124	0
Na-naphthol(4)sulphonate.....	215	0	139	0	167	0
Naphthalenepropionic acid*.....			237	0	385	0

\* Solubility approximately 0.003 at room temperature.

tree, using three branches for each concentration of each compound. It was necessary in some of the treatments to include several smaller trees, although in such cases comparable concentrations of each compound were grouped on the same tree.

The use of trees outdoors necessitated protecting the flowers against pollination by insect or other means. Large glassine bags were fastened over the branches just prior to blossoming. Six days later the bags were removed long enough to count and spray the opened flowers and to remove all unopened buds. The bags were then replaced until all danger of fertilization had passed.

Naphthaleneacetic acid, shown in previous work to be very potent in effecting parthenocarpy in holly, was included in the tests of both greenhouse and outdoor trees. In both cases, and in the three concentrations used, it was somewhat excelled by naphthalene acetamide. The acetamide, in addition to being more potent, has the advantage of not producing any observable nastic disturbances of leaves or petioles. We have often observed the comparative inactivity of this compound in nastic responses with a number of plants.

Of particular interest is the relative ineffectiveness of the potassium salt of naphthaleneacetic acid. Although it brought about an initial swelling of the holly ovaries, the color of the ovaries and of the pedicles turned progressively yellow instead of acquiring the deep green hue of those treated with naphthaleneacetic acid, and most of the young fruits soon abscised. At present no explanation is offered for the low activity in parthenocarpy of the potassium salt which, in various other plant responses, has been reported of high activity. The difference in pH between a solution of the acid and of the potassium salt and the probable resulting difference in cell penetration, however, should be considered in view of the work of ALBAUM, KAISER, and NESTLER (1). They showed that the penetration of indoleacetic acid into *Nitella* cells takes place more rapidly at low pH values of the external medium than at high ones, and attributed the results to the effect of high hydrogen ion concentration depressing the dissociation of indoleacetic acid which, evidence indicates, enters the cells in the non-dissociated form. It seems hardly necessary to state that the greater activity of naphthaleneacetic acid than of the potassium salt was not due to its acidic nature causing release of naturally occurring bound auxin. Possibly it should be recorded that acetic acid, in a range of concentrations, did not stimulate parthenocarpy when sprayed on holly flowers.

In view of the high activity of naphthalene acetamide, it is curious

that its thio compound should be of such low effectiveness. No data are available on the relative dissociation of these compounds which might throw light on the reason for their different effectiveness.

Phenylacetic acid, while not rated high in activity in other plant responses, gave no indication whatever of any stimulation in these tests. This was also the case with sodium naphthol(4)sulphonate, which was included because it is one of the compounds reported by TRAUB (4) to have a marked stimulating effect on rooting of *Passiflora* and *Bignonia* cuttings, but to be without effect on *Avena* curvature.

With respect to the nature of the action of these substances in stimulating fruit development, little can be said. It appears that they prevent the formation of an abscission layer at the base of the flower pedicle, thus permitting the flow of nutrients necessary for growth of the fruit and perhaps substituting in part for the stimulus set up by the developing fertilized ovule. At least the subsequent histological development of the parthenocarpic fruits has been shown to parallel that of normal, pollinated ones (2).

As more data become available on the effectiveness of many compounds in various plant responses, such as curvatures of stem or leaf, elongation, root production, gall formation, parthenocarpy, etc., it becomes increasingly evident that the order of effectiveness in one response may be very different in terms of some other response, and that "relative activity" of a compound is without meaning except with reference to the particular test method and probably also to the particular plant used.

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## ADOLF CARL NOÉ

In the death of Dr. ADOLF CARL NOÉ on April 10, 1939, American geology and paleobotany have lost a devoted leader. Dr. NOÉ was born in Austria on October 28, 1873, and educated in part at the universities of Gratz and Göttingen. During his student days at Gratz he developed an interest in paleobotany, and was a demonstrator in paleobotany in that institution from 1895 to 1897. On coming to America, he filled several positions in the teaching of modern languages and literature. He received an A.B. degree at Chicago in 1900, and the Ph.D. in Germanic Languages and Literatures in 1905. He was a member of the staff of this Department until 1923. In that year he transferred to the Departments of Geology and Botany, where he remained for the rest of his life. He came into contact with many students in these departments during these years.

In the field of paleobotany he distinguished himself by discoveries of coal balls in the American coal fields, by becoming one of the most active and enthusiastic collectors of paleobotanical material, and by developing new methods of studying paleobotanical materials, especially refinement of the nitrocellulose peel method of making microscopic sections of coal balls.

Dr. NOÉ was associated with various state geological surveys, particularly in Illinois, Iowa, and Kentucky. Among his scientific contributions were several books: *The Fossil Flora of Northern Illinois*; *Ferns, Fossils, and Fuels*; a textbook on coal, and numerous scientific papers. He was a member of many learned societies here and abroad, and was honored and decorated for his scientific and humanitarian services after the World War. He was a man of kindly spirit, with a keen sense of humor. He is much missed by his students and colleagues.

E. J. KRAUS



## BRIEFER ARTICLE

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### POLYEMBRYONY IN MYRCIARIA CAULIFLORA

(WITH ONE FIGURE)

The jaboticaba, *Myrciaria cauliflora*, was introduced to this country from Brazil a few decades ago, and in more recent years trees of it have fruited in Florida. This may be a promising new fruit crop for Florida and possibly also for other subtropical regions of continental United States.

Dissection of twenty-five sprouted seeds has revealed that polyembryony is usually encountered. In the material used the number of seedlings of varying sizes from single seeds ranged from one to six; the usual number was two. When more than three embryos were present in a single seed, some of them were tiny. Figure 1 (p. 234) shows one, two, and three seedlings sprouting from single seeds. Under the usual nursery conditions only the larger seedlings survive from a single seed, usually only two.

Morphological studies to trace the origin of the supernumerary embryos are under way.—HAMILTON P. TRAUB, *Division of Fruit & Vegetable Crops and Diseases, U.S. Department of Agriculture, Orlando, Florida.*

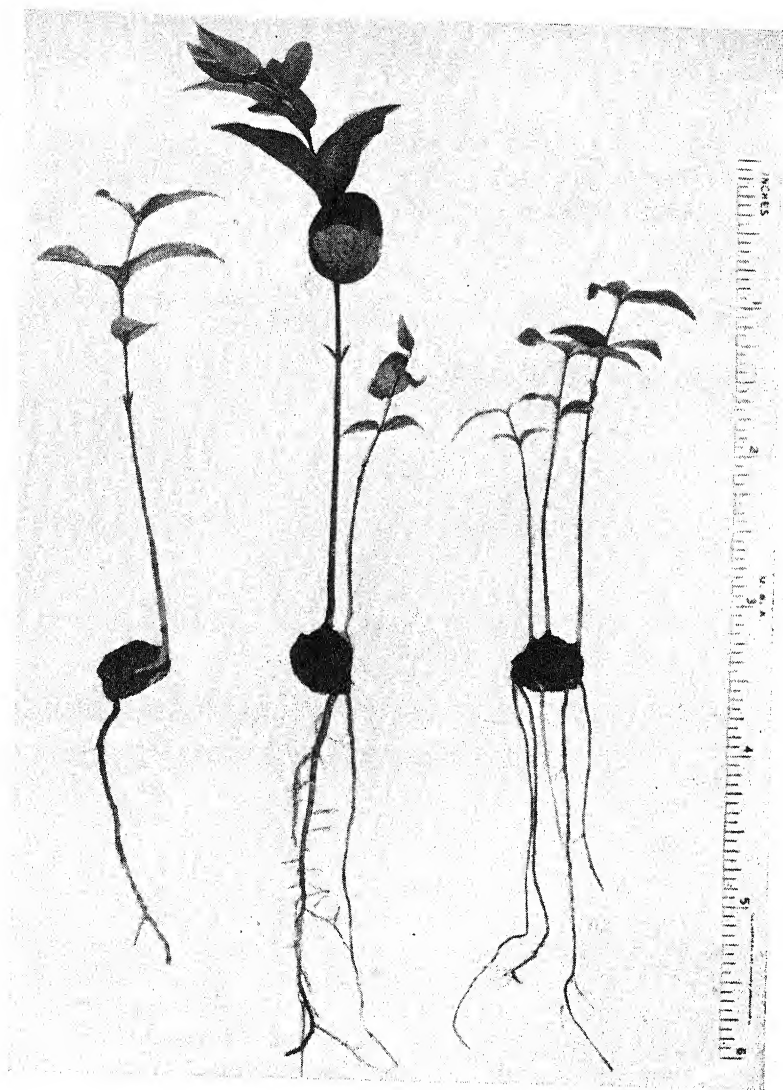


FIG. 1.—Sprouting seeds of *Myrciaria cauliflora*, showing one, two, and three sprouting embryos per seed.

## CURRENT LITERATURE

*Le genre Mycena. Étude cytologique et systématique des espèces d'Europe et d'Amérique du Nord.* By ROBERT KÜHNER. Encyclopédie mycologique X. Paris: Paul Lechevalier, 1938. Pp. 710. Illustrated. 300 fr.

According to the author, about one-sixteenth of the agarics of Europe are included in the genus *Mycena*, and it may be assumed that in temperate North America the proportion is substantially the same. The fructifications of the majority of the species are small and ephemeral, and the descriptions of the older mycologists emphasize to too great an extent superficial and often essentially secondary or inconstant characters. These facts have combined to make this one of the most difficult genera of the Agaricaceae from the taxonomic point of view. In the present volume an attempt is made to consider all European species and as many North American species as possible, using what is largely a new arrangement based primarily upon the microchemical reaction of the spores and hyphae to iodine, and stressing such microscopic features as cystidia and the surface cells of pileus and stipe. Where the original descriptions are inadequate and authentic specimens have not been available, it has been impossible to fit the species concerned into the author's system; but full notes, including translations of the original descriptions, are given for all such forms. Seventeen species, three varieties, and eight forms are described as new, several of them being attributed jointly or solely to R. MAIRE, whose notes were turned over to the author. Considering the scope of the work, this would seem to suggest a conservative approach.

While mainly taxonomic, nearly a quarter of the text is concerned with more general matters, including technique, anatomy, morphology, and cytology. Much of this, particularly the discussion of so-called sexual phenomena, is of general interest.

Only experience can determine the extent to which the taxonomic subdivisions proposed by the author will prove to be applicable to the North American species, but the detailed original studies and the careful compilation of previous information make this volume a new starting point for the study of the genus directly concerned and furnish abundant suggestion for a similar approach to other genera of the Hymenomycetes.—G. W. MARTIN.

*The British Islands and Their Vegetation.* By A. G. TANSLEY. Cambridge, England: University Press; New York: Macmillan Co., 1939. Pp. xxxviii+930. Illustrated. 45/.

Ecologists and all interested in the natural and semi-natural vegetation of the British Islands should be delighted with this masterly and comprehensive description by the one best qualified to present it. The book is a modern out-

growth of the earlier volume, *Types of British Vegetation*, published in 1911, which did much to stimulate the research embodied in many of the papers on British plant communities appearing in the *Journal of Ecology* over the last quarter of a century, upon which the present volume draws very largely. Professor TANSLEY's purpose, admirably fulfilled, has been to weld this material "into a continuous story which can be read, and not merely "consulted," by the student—British or foreign—who wants trustworthy information about the British plant communities, but who is not an ecological specialist." The resulting treatment is therefore somewhat longer, more explanatory of concepts and terms, and more repetitious than if intended primarily for ecologists. There are very few of the latter, however, excepting those completely unsympathetic with Professor TANSLEY's ecological philosophy, who will not derive both pleasure and profit from the orderly way in which he has marshalled and integrated the facts (possibly already familiar) in accord with it. The treatment contains little of phytosociological nomenclature (of BRAUN-BLANQUET), or of bioecology, although a chapter on the "biotic factor" is included and many effects produced by animals on plant communities are recorded. It is in complete agreement with the dynamic concepts previously developed by Professor TANSLEY in his books and theoretical papers.

The volume is divided into nine parts: the British Islands as environment of vegetation (146 pp.); history and existing distribution of vegetation (64 pp.); nature and classification of vegetation (30 pp.); the woodlands, excluding plantations of exotic trees (244 pp.); the grasslands, including all permanent planted pastures of native species as well as "natural" grassland formed from grazing other vegetation types (92 pp.); the hydroseres, including freshwater, marsh, fen, and bog vegetation (144 pp.); heath and moor (52 pp.); mountain vegetation (41 pp.); and maritime and sub-maritime vegetation, excluding intertidal seaweed communities on rocky coasts but including salt-marsh (89 pp.). There is an understandable prominence of data from southeastern England, where many of the modern detailed studies have been made.

An adequate index is provided, and pertinent references follow each of the forty-three chapters. The book is graphically and beautifully illustrated by 179 text figures and 162 plates containing 418 photographs. It should be required reading for all serious students of ecology anywhere, and for any American botanists who feel that the present descriptions and summaries of the vegetation of the United States or any of its larger subdivisions are at all adequate or complete.—C. E. OLMSTED.

*German-English Botanical Terminology.* By HELEN and ERIC ASHBY, HAROLD RICHTER, and JOHANNES BÄRNER. London: Thomas Murby & Co., 1938. Pp. xi+195. 10/.

The reader will find much that is fundamental in botany as well as achieve a vocabulary and a method of expression in German from the use of this novel

and excellent book. Textual material reads smoothly, connectedly, and easily in English on one page and on the facing page there is as nearly a literal translation in German as is feasible, considering the difference in method of expression and phraseology of the two languages. Chapters deal with morphology, phylogeny, cytology and genetics, physiology, ecology, and pathology in a gratifyingly competent way, considering the brevity of the book. There are also appendixes giving the common names of wild and cultivated plants, names of the more important plant diseases, abbreviations used in English and German botanical literature, and an index in each language. The book does not take the place of an extensive dictionary, but it does far more than any dictionary can by giving the flow and sense of written language which a reader may desire to interpret.—E. J. KRAUS.

*Clés des Mucorinées.* By N. A. NAUMOV. Encyclopédie mycologique IX. Paris: Paul Lechevalier, 1939. Pp. xxxvi+137. Illustrated. 100 fr.

Translated from the second Russian edition by S. BUCHET and I. MOURAVIEV, the French text incorporates additional material contributed by the author. A brief introduction discusses the morphology of the fungi constituting the order, but the bulk of the volume is in the form of a detailed key to the suborders, families, genera and (under each genus) to the species recognized. A very complete index lists, together with recognized genera and species, synonyms and rejected names not mentioned in the text. Since H. ZYCHA's monograph (*Kryptogamenflora der Mark Brandenburg* VIa, 1935) appeared almost simultaneously with the Russian text, an appendix is devoted to a consideration of the divergences between the two treatments. NAUMOV does not include the Endogonaceae, but even so recognizes approximately half as many species again as does ZYCHA. One new genus, *Protoabsidia*, and a number of new species are indicated, without Latin diagnoses. The striking American genus *Mycotypha*, published in 1932, has not yet found its way into European monographs.

While the present work will not replace ZYCHA's excellent and logical treatise, it constitutes a valuable supplement to it. Together they should provide needed impetus to the study of these highly significant and important fungi.—G. W. MARTIN.

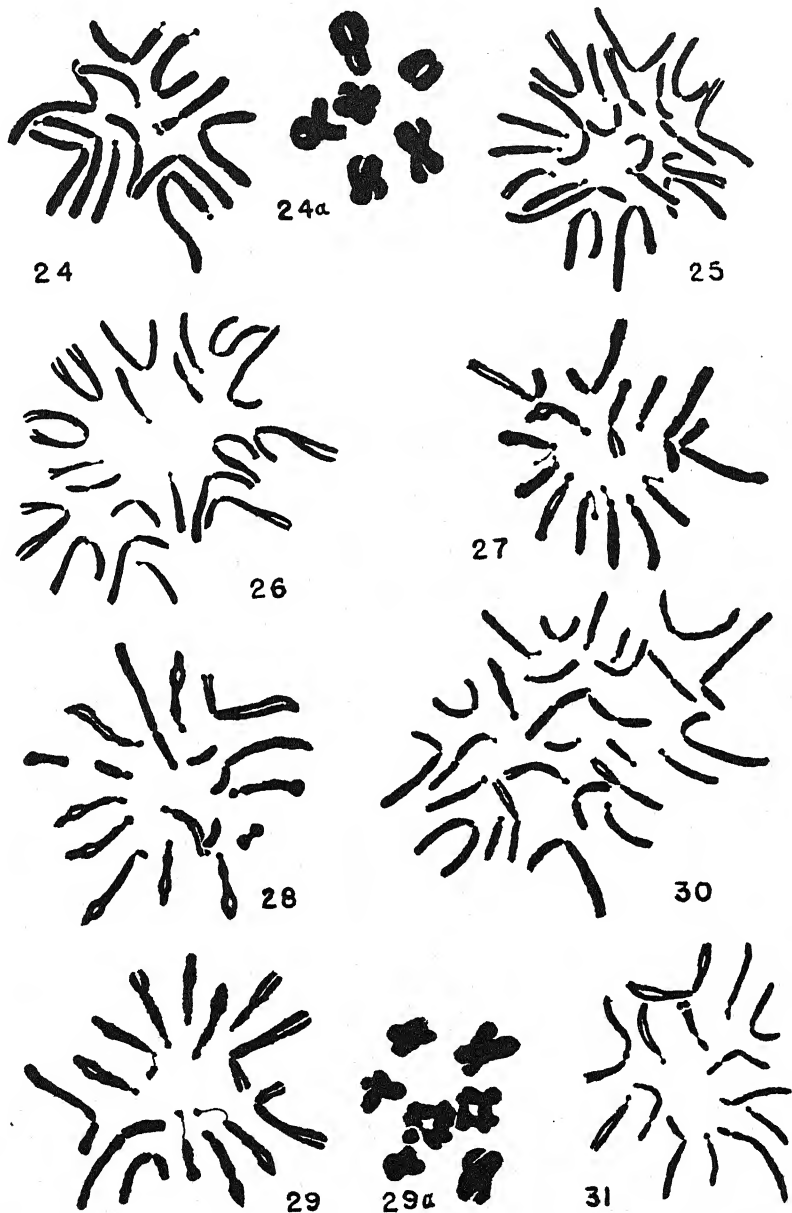
*Introduction to Floral Mechanism.* By S. G. JONES. Glasgow: Blackie & Son, 1939. Pp. xi+274. Figs. 71.

A generalized presentation of floral morphology, cytology, genetics, and plant breeding designed to acquaint the elementary student with these subjects. The text is direct, largely descriptive, and presents much detail, but in some instances the morphological homologies are not clearly drawn nor critically stated. The illustrations are numerous, well drawn, and though lacking in many details essential to critical morphological studies and interpretation, no doubt serve the purpose intended for the elementary student.—E. J. KRAUS.

*Bibliography of References to the Literature on the Minor Elements and their Relation to Plant and Animal Nutrition.* Originally compiled by L. G. WILLIS. Chilean Nitrate Educational Bureau, Inc., 120 Broadway, N.Y., 1939. Pp. 488. \$1.00.

The third edition of this valuable annotated bibliography contains 4628 abstracts dealing with the elements used and needed in small amounts in the nutrition of plants, animals, and man. The number of authors abstracted is about 2200. The elements are arranged in alphabetic order, and the authors also alphabetically under each element included. It is interesting to note that 53 of the 92 naturally occurring elements are listed in the index. Not all of these are certainly required by any one species of plant or animal, nor is the inclusion of an element in the list to be construed as indicating usefulness. If they have been investigated, they are included in the list.

The first edition, published in 1935, included just over 1800 abstracts; and the second edition in 1937, almost 2800. These earlier editions were less complete than the present one, nevertheless the increasing number of citations is an index of the extraordinary interest in the trace elements at the present time. The activity in the field will undoubtedly continue, since we know very little yet of the physiological functions of any of these elements. The Chilean Nitrate Educational Bureau deserves commendation for the enlightened policy which established and maintains a publication so vital to research on plant nutrition. Every student of nutrition will find this vast storehouse of information an indispensable adjunct to his work.—C. A. SHULL.



# BEAL—CALOCHORTUS

Corrected reproduction, to be substituted for the incorrect one shown on p. 538 of the issue of March, 1939.



# THE BOTANICAL GAZETTE

December 1939

HENRY CHANDLER COWLES

(WITH PORTRAIT)

HENRY CHANDLER COWLES was born at Kensington, Connecticut, on February 27, 1869. He was graduated from Oberlin College with the degree of A.B. in 1893, and the next year taught natural science at Gates College. In 1895 he was given a fellowship at the University of Chicago, beginning his graduate studies in geology; but later he became a member of the first group of students in the newly organized Department of Botany of this Institution, and an assistant in that Department in 1897. He received the degree of Doctor of Philosophy in 1898, presenting as his thesis his classical paper *The Ecological Relations of the Vegetation on the Sand Dunes of Lake Michigan*. Application of his concepts concerning dynamic vegetation to vegetation in general resulted in his *Physiographic Ecology of Chicago and Vicinity*, a philosophy relating to vegetation in which the central principle was that classification to be valid must be genetic and dynamic. For the first time the concepts of succession and climax were adequately expressed. The principles expressed have stood the test of time and have formed the basis and point of departure for the dynamic concept of ecology.

In the Department of Botany at the University of Chicago DR. COWLES was repeatedly advanced in rank, becoming Professor in 1911 and Chairman of the Department in 1925. In 1926 he became Editor of the BOTANICAL GAZETTE. To both tasks he gave a full measure of devotion and energy until his retirement in 1934.

In 1911 appeared the *Chicago Textbook of Botany*. In the volume dealing with ecology DR. COWLES stressed the theory of mechanical causation rather than of teleology and adaptation. In 1914 the Eco-

logical Society of America was organized, largely through his efforts and those of his former students. He was its first secretary-treasurer, its president in 1917, and always a wise counselor regarding its welfare. In 1935 the July issue of *Ecology*, filled with articles from students and colleagues throughout the world, was dedicated to him by his students and friends. His alma mater, Oberlin College, conferred upon him the honorary degree of Sc.D. in 1923.

For many years DR. COWLES was president of the Chicago Academy of Sciences, a charter member and an active supporter of the Illinois State Academy of Science, and also a patron and trustee of the Geographic Society of Chicago and president of the Society for a term of years. A member of many other scientific societies, he served as president of the Association of American Geographers in 1910; as president of the Botanical Society in 1922; and vice-president of Section G of the American Association for the Advancement of Science in 1913. In 1930 he was made president of the section of phytogeography and ecology of the International Botanical Congress which met at Cambridge, England.

As a leader in plant science, particularly in dynamic plant ecology, DR. COWLES was enthusiastic but never dogmatic. From the beginning of his career to his retirement there was always gathered about him a group of men and women who aided in augmenting and refining his concepts and in spreading the knowledge of dynamic ecology.

No teacher brought his students more directly to nature than he. Much of his teaching was done on field trips, and he encouraged others to use the same classroom. As a scientist and as a teacher he inspired his colleagues and students in the quest of accomplishment. But in the hearts and minds of his associates there was the love for him personally, as great as the respect for his scientific attainment. Unfailing good humor he had in abundance. To live and to work intimately with him was to come to know and appreciate his sense of rightness, of justice, and his depth of understanding.

HENRY CHANDLER COWLES passed away at his home, September 12, 1939. His achievements laid the foundation for a new and useful branch of science. Through professional and personal contacts he had a multitude of devoted friends. The impress of his having lived is deep in the field of the science he served and in the minds of those who knew him.—E. J. KRAUS.

# NUTRITION OF THE MYXOMYCETES. I. PURE CULTURE AND TWO-MEMBERED CULTURE OF MYXOMYCETE PLASMODIA<sup>1</sup>

ARTHUR L. COHEN

(WITH FOUR FIGURES)

## Introduction

The Myxogastreales (myxomycetes) and their probable congeners, the Acrasiales, have long attracted attention because of their position at the boundary of the plant and animal kingdoms. They are particularly interesting in their faculty of holozoic nutrition, coupled with the formation of definitely organized fruiting bodies—the latter a plantlike character. The myxomycetes possess the additional experimentally valuable property of forming large ameboid plasmodia, of indefinite extent and of relatively unspecialized nature.

In view of their interest it is surprising that nearly all, if not all, studies on the myxomycetes have been made with contaminated plasmodia, and many of these observations were made somewhat cursorily on plasmodia brought in directly from the field, not too well freed of their natural substrates, and under uncontrolled conditions.

Controlled, reproducible laboratory cultures are of great value for the experimental study of the myxomycetes no less than for other organisms. For extensive nutritional studies, controlled cultures are a *sine qua non*. The work reported in this paper has been on the preparation and maintenance of controlled cultures for the studies on nutrition of the myxomycetes now being carried on in this laboratory.

## Literature review

GENERAL OBSERVATIONS.—In spite of the criticism of past work just mentioned, students of the myxomycetes have been able to make cogent observations on the feeding habits of myxomycete

<sup>1</sup> Contribution from the Laboratories of Cryptogamic Botany and the Farlow Herbarium, Harvard University, no. 174.

plasmodia and were often able to draw valuable inferences. The first of these observations were made by CIENKOWSKI and DEBARY.

The ingestion of solid particles was recorded by CIENKOWSKI (11) and DEBARY (1, 2, 3). Neither could decide whether the chief means of feeding was by the ingestion and assimilation of solid food or by the absorption of dissolved nutrients through the plasma membrane.

That some myxomycete plasmodia can live wholly on ingested particulate matter was shown by LISTER (21). He refers to the "parasitism" of *Badhamia utricularis* on hymenophores, particularly *Stereum hirsutum*. He observed the dissolution of the hyphae ingested by the plasmodium as well as a similar destruction of starch grains previously swollen by moist heat. HOWARD and CURRIE (17, 18) extended the observations of LISTER and others to include the mycelia and fruiting bodies of a number of fungi. The several workers speak of the destruction of mycelial fungi by myxomycetes as parasitism, but perhaps such speedy and complete dissolution of the "host" should better be called preying.

ČĚLAKOVSKÝ (9), extending the work of PFEFFER (27), studied the ingestion and digestion of living and non-living particles by plasmodia. He found that food vacuoles formed around particles which could be digested (egg white, starch, green algae, and vegetable cells), while generally those which could not be digested (fungus spores, living cells of *Euglena*, *Spirogyra*, etc.) were not surrounded by vacuoles. He found no evidence, in limited observations, of the digestion of the bacteria (*Bacillus megatherium* and *B. subtilis*), although the bacteria were surrounded by vacuoles. However, the ingestion and digestion of bacteria by swarm-spores of myxomycetes, if not by plasmodia, had been seen by LISTER (22).

REPORTS OF TWO-MEMBERED CULTURE.—That bacteria play a major role in the nutrition of the Acrasiales and therefore probably also in the nutrition of the myxomycetes, was shown by NADSON (25). NADSON was the first to claim pure culture of at least a myxomycete-like organism, *Dictyostelium mucoroides*. His bacteria-free cultures developed irregularly and finally died. Other cultures made in association with *Pseudomonas fluorescens liquefaciens* were normal. Although NADSON incorrectly considered the relation between

the bacteria and the *Dictyostelium* to be symbiotic (cf. RAPER, 30) rather than two-membered,<sup>2</sup> his work indicates a careful technique, evinced by the rigid methods he employed in determining the purity of his cultures. NADSON showed that mere macroscopic examination of culture plates was completely unreliable, and that the real tests for purity depended on careful microscopic examination and on inoculations into media particularly favorable for the development of bacteria. We find no examples of equal care reported in the literature on the "true" myxomycetes until the work of VON STOSCH (37). For the further work on the Acrasiales, the reader is referred to RAPER'S (31 *et prev.*) reviews and researches.

A definite indication that the true myxomycetes can derive a large part of their nourishment by the ingestion of cells is given by HENNEBERG (14). He fed yeast to the myxamebae and plasmodia of *Physarum leucophaeum* and was able to observe the intake, and often the subsequent ejection, of the yeast by the myxamebae. This egestion of cells after ingestion caused HENNEBERG to conclude that the slime sheaths or metabolic products secreted by the yeast were more important nutritionally than the cells themselves.

HENNEBERG'S paper was followed by a similar one by CHRZĄSZCZ (10), who interpreted the feeding in an altogether novel manner. According to CHRZĄSZCZ, the myxamebae and the yeast are in competition for the same medium, and the myxamebae attack and ingest the yeast cells merely to destroy them, the killed cells being later voided or perhaps digested for subsidiary nutrition.

That definite two-membered cultures of plasmodia and bacteria could be established was first proved by PINOY (28, 29). He sowed the spores of *Didymium difforme* and *D. effusum* on agar slants and found that some of the cultures remained altogether clear and without development of any sort. But those cultures in which bacteria also developed exhibited a vigorous growth of the plasmodia of these

<sup>2</sup> The term "two-membered culture" is used as a literal translation of the German *zweigliedrige Kultur* employed by protozoologists. *Zweigliedrige Kultur* has the advantage over such phrases as pure-mixed culture and association culture by being specific in meaning—the feeding of one living organism upon another which in turn thrives upon the supplied substrate.

"Pure culture" is employed here in its strict sense: a culture in which only one kind of living organism is present.

myxomycetes, and best results were obtained with *Bacillus luteus* as the second member of his two-membered cultures. PINOY concluded that the bacteria were used directly as food by the plasmodia.

Later VOUK (39) cultivated *Didymium nigripes* for 2 years in two-membered culture with a bacterium, probably *Pseudomonas fluorescens liquefaciens*. He was unable to obtain plasmodia in a state of purity and doubted whether it were possible to grow them so. In agreement with other workers, he found that media which allowed too rich growth of the bacteria were unfavorable for the cultivation of plasmodia. VOUK made an important technical contribution which seems to have escaped the notice of succeeding researchers; namely, that bacteria which accompanied the plasmodia formed neither well defined nor even diffuse colonies on the agar, but always were to be found in numbers at the margins of the plasmodial strands, at the periphery of the plasmodial front, and in the etched tracks left by the plasmodium as it crawled over the agar.

In 1928 SKUPIENSKI (34) reported the two-membered culture of *Didymium difforme* with *Aspergillus glaucus*, *Torula glutinis*, *Sarcina lutea*, *Bacillus prodigiosus*, and particularly vigorous cultures with a bacterium resembling *B. vulgaris*. SKUPIENSKI found no growth of the plasmodia in absolutely pure culture, and concluded that micro-organisms were necessary for growth of this myxomycete. SKUPIENSKI had earlier (32) claimed the pure culture of *Didymium nigripes*.

In his work with *D. nigripes* SKUPIENSKI sowed the spores on the walls of tubes containing agar slants. The small plasmodia formed from the germinated spores crawled down from the glass to the agar, presumably leaving the bacteria behind. Normal growth and fructification occurred on the clear agar, this clarity apparently being accepted as complete proof of non-contamination.

VON STOSCH (37) cultivated plasmodia of several species of myxomycetes, especially *Didymium nigripes*, in two-membered cultures with *Torula* sp., beer yeast, an unknown mold, and several species of bacteria. After several transfers the vitality of the plasmodia declined, with the exception of *D. nigripes* in two-membered culture with a yellow micrococcus. Even here VON STOSCH believed the bacterial colonies to be composed of two species, not separable on hay

agar plates. Only negative results were obtained with attempted pure cultures on bacteria killed with heat or chloroform.

Finally WATANABE (40, 41) tested the ability of a wide selection of plasmodial species to grow on various bacteria and yeasts. Since the micro-organisms were streaked on plain washed agar, this is the first clean-cut case of the ingestion and assimilation of micro-organisms in the absence of other supposititious dissolved nutrients. Unfortunately WATANABE does not mention whether he grew the plasmodia through several serial transfers, and therefore the possibility of continued two-membered cultures of his plasmodia is left in doubt.

REPORTS OF PURE CULTURE.—Several workers (CONSTANTINEANU, SKUPIENSKI, CAYLEY, *et al.*) have grown myxomycetes in solutions or agars in which no particulate matter was intentionally supplied. A misinterpretation of their results has occurred in the literature; namely, that plasmodia can grow on dissolved nutrients alone. Indeed MACBRIDE and MARTIN (23, p. 7) state: "Several species have been grown in pure culture; among others, *Didymium difforme* (SKUPIENSKI, 1926, 1927, 1928) and *Didymium nigripes* (CAYLEY, 1929)." Yet on examination, the papers of SKUPIENSKI and CAYLEY give no basis for such a belief. For example, SKUPIENSKI says nothing concerning culture in his 1927 paper (33).

"Les spores sont toujours accompagnées par les bactéries qui se multiplient rapidement et servent de nourriture aux zoospores, myxamibes, et plasmodes" (33, p. 150).

"Cette espèce [*Didymium difforme*] vit en association symbiotique avec une Bactérie rapprochée de *Bacillus vulgaris*. Sans cette dernière son développement est très faible et n'arrive pas jusqu'au bout" (34, p. 327).

"... les spores germent au bout de quelques heures après leur ensemencement. Les bactéries, associées au Myxomycète, se développent avec la même intensité" (35, p. 205).

CAYLEY states: "No serious attempts have been made to eliminate bacteria from the cultures; in fact it is almost impossible" (8, p. 228).

The following abridged statement is from CONSTANTINEANU: "In Myxomyceten-Kulturen . . . die sich ansammelnden Stoffwechsel-

produkte teils von Bakterien, teils von Myxomyceten selbst her-rühren, ohne dass es bisher möglich wäre, beide zu unterscheiden'' (12, p. 525).

Of the two claims to pure culture made in the literature, SKUPIENSKI's has already been considered. HOWARD's contribution to the culture of the myxomycetes appeared in 1931. HOWARD's method (16) and CAMP's modification of it (7) made it possible to grow certain plasmodia in as great a quantity as desired. HOWARD makes no claim to pure culture, but states that after several transfers through non-nutrient plates the plasmodia "will be reasonably free of contaminating organisms and may then be transferred to oat agar."

In another paper (15) of the same year, HOWARD speaks of uncontaminated plasmodia growing well on plugs of sterilized *Polyporus frondosus*. However, the section of the paper called "food relations" leaves one in doubt about the purity of the cultures, owing to the seemingly contradictory statements made about the presence of other organisms.

From the foregoing review it is evident that there are only a few, probably doubtful, examples of true two-membered cultures (VOUK, PINOY, SKUPIENSKI, and VON STOSCH); and only two questionable instances of absolutely pure culture (SKUPIENSKI, HOWARD).

It seemed to the writer, therefore, that the controlled culture of these organisms should be studied in detail and adequate criteria of purity established. The results of the study are presented in this report.

### Materials and methods

Most investigators used spore cultures to obtain uncontaminated plasmodia. The use of spores has the disadvantages of tediousness and of being limited to members of the genus *Didymium* and the few other myxomycetes which form plasmodia readily in culture. With the exception of *D. squamulosum*, therefore, which was regularly obtained from spore cultures, all the myxomycetes studied (*Badhamia foliicola*, *B. utricularis*, *Fuligo septica*, *Stemonitis axifera*, and the yet unidentified "plasmodium 51") were obtained as plasmodia or sclerotia in the field, and cultivated by a modification of HOWARD's

(16) method to insure a plentiful supply of material with which to attempt pure culture.

One of the two pure culture methods used by the writer was suggested by the work of WATANABE (40, 41) previously mentioned. Although the "enrichment" method of the writer is founded on WATANABE'S work, it was seen later that such two-membered cultures had been used for amebae by BEIJERINCK (4, 5) and thoroughly investigated as a method for the pure culture of amebae by OEHLER (26).

For purification itself, two methods were employed:

1. The migration method, in which plasmodia were allowed to crawl over several plates of non-nutrient agar.
2. The enrichment method, in which plasmodia were fed washed, living yeast cells in the form of a heavy suspension streaked on non-nutrient agar.

The writer has used and now suggests the following two criteria of pure culture for myxomycete cultures so obtained.

1. There must be no growth of micro-organisms in nutrient media inoculated from the cultures. The media should include at least one comparable in composition with the media used for pure culture of plasmodia, of approximately the same pH, and incubated at the same temperature for a sufficiently long time.
2. The cultures must be carried through several transfers without decline in vigor (13), and strictly should grow indefinitely on the pure culture media without a permanent loss of vigor.<sup>3</sup>

When employment of these criteria showed a plasmodium to be pure, it was inoculated into oatmeal agar simultaneously with a pure culture yeast suspension for the two-membered cultures; and into non-nutrient agar streaked with autoclaved bakers' yeast for the pure cultures.

The following media were employed, and are referred to by the assigned symbols in the detailed accounts of the outlined methods.

<sup>3</sup> Under conditions where factors such as temperature, humidity, and size of the inoculum are not readily controlled, it may be expected that cultures will show temporary changes in vigor. In the writer's cultures fluctuations are exhibited by *Physarum polycephalum* particularly, and by two-membered cultures in which the yeast grows faster than the plasmodium.

### Media<sup>4</sup>

#### YEAST-EXTRACT DEXTROSE BROTH (YED)

Yeast extract (Difco, powdered) . . . . .	2.5 gm.
Dextrose . . . . .	10.0
Na <sub>2</sub> HPO <sub>4</sub> ·12H <sub>2</sub> O (Merck's c.p.) . . . . .	1.4
Tap water . . . . .	1 liter

This broth, dispensed into tubes, is used to test for contaminants in cultures. For growing the yeast utilized in the experiments the broth is modified by leaving out the disodium phosphate. Agar (2 per cent) is added for plates and for stock pure-culture slants of sugar-requiring micro-organisms.

#### YEAST-EXTRACT BROTH (YE)

This is similar to the preceding but without phosphate or dextrose. It is used in testing for contaminants in cultures and with 2 per cent agar for growing stock cultures of the bacteria employed.

#### AUTOCLAVED YEAST (YA)

Equal quantities by weight of bakers' yeast and distilled water are thoroughly mixed in a mortar and dispensed into tubes in 5-cc. quantities for autoclaving and storage. Before use, the tubes are gently agitated to suspend the yeast evenly. This medium is used for the pure culture of myxomycete plasmodia.

#### LIVING YEAST (YL)

The yeast is cultured in liter flasks, each containing 200 cc. of YED without phosphate, and the flasks are occasionally shaken for aeration. At the end of 48 hours the flasks are inclined until the yeast settles, and the excess medium decanted off through a flame. Sterile distilled water (about 50 cc. per flask) is added, the flasks well shaken, allowed to stand until the yeast again settles, and the washing repeated twice more. The final suspension is centrifuged and only a volume of water equal to the volume of the yeast layer is left. The tubes are stored until needed, preferably not longer than a week, at 6° C. This yeast is streaked on sterile WA plates of pH 6.0 for enrichment plates.

<sup>4</sup> All media are sterilized by autoclaving 20 minutes at 15 pounds' pressure, unless otherwise indicated.

The technique described is for *Saccharomyces ellipsoideus* strain 8.2.1 C.B.S. (*Centraalbureau voor Schimmelcultures*). In all cases where C.B.S. cultures are mentioned the strain numbers are those of the Hopkins Marine Station from which the writer obtained all his C.B.S. strains. For very slimy or slow-growing yeasts, the technique is modified by longer period of incubation, solid substrates, heavier centrifuging, etc.

#### UNWASHED AGAR OF PH 6.0 (6.0 UA)

Agar (Difco Bacto, granulated).....	20 gm.
$\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ .....	0.96
$\text{KH}_2\text{PO}_4$ .....	1.45
Distilled water.....	1 liter

This agar has a pH stable near 6.0 even after repeated melting and pouring. The molar ratio  $\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$  is 8/2, and the molar quantity is M/75 total  $\text{PO}_4$ . Slants of this agar, streaked with YA, are used for growing pure cultures of myxomycetes.

#### WASHED AGAR (WA)

One hundred gm. of granulated Difco Bacto agar are washed for 5 days with frequent changes of distilled water. After the last wash, as much water is decanted off as possible and the agar placed in a fine cheesecloth which is squeezed until all free water has been driven out. The pearly white, friable mass is weighed and stored in the refrigerator until needed. With 100 gm. dry agar the final weight should be between 500 and 600 gm. Two per cent agar (dry weight basis) is prepared and buffered in the same manner as the 6.0 UA. Because of the large amount of water it is best to subtract the weight of the washed agar from that of a liter of water so that the final weight is 1000 gm.

For any required pH, sample flasks are made up with 2 per cent agar and buffered by different ratios of  $\text{Na}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ , the total added phosphate always being M/75 in the final preparation, pH's of the samples taken after autoclaving (pH's may be changed by autoclaving), a curve of pH against  $\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$  drawn, and agars of proper pH prepared by using the values interpolated on the curve.

Washed agar is used for migration and enrichment plates since unwashed agar has enough organic substance to support the growth of contaminants (fig. 2C).

#### OATMEAL AGAR (OA)

Quaker Rolled Oats is dispensed into 125 ml. Erlenmeyer flasks to an even layer one grain in thickness, and 20 cc. 6.0 UA is poured into each flask. Just sufficient oatmeal is used to allow some of the grains to reach the surface after autoclaving (dispensing oatmeal agar into flasks was found to be unsatisfactory owing to the settling of grains while the agar was poured). This OA is used for stock two-membered cultures of plasmodia.

GLASSWARE.—In these experiments only two kinds of special glassware are employed, the small calibrated pipettes and the vial-capped centrifuge tubes, and they are sterilized in dry heat by the usual bacteriological procedure.

The small pipettes for streaking the autoclaved and living yeast are prepared by drawing out 30-cm. lengths of 5-mm. exterior diameter soft glass tubing in the middle, and breaking. Best results in streaking are obtained with long slender nozzles, the tips of which are 1-1.5 mm. exterior diameter. These small pipettes are calibrated by marking at every centimeter with acid-and-heat-resistant paint (each centimeter corresponds to 0.05 cc.). They are subsequently sterilized in large, plugged test tubes.

Small (13×100 mm.), rimless, pyrex culture tubes, capped with inverted shell vials, are used for aseptic centrifuging of yeast cultures. The use of shell vials instead of cotton plugs avoids the objectionable accumulation of lint in the centrifugate.

COLLECTION AND GROSS CULTURE.—Collected plasmodia are generally heavily contaminated and rather small. Preliminary to purifying, therefore, each plasmodium was partially cleansed of contaminants by allowing it to crawl over one or two plates of 2 per cent agar before attempts were made at culture. A modification of HOWARD'S (16) oatmeal agar method was used for culture.

Paper toweling or filter paper was spread over the bottom of a moist chamber, wetted, the excess water allowed to drain off, and the plasmodium-bearing substrate placed at one side of the dish. As the

plasmodium crept over the paper, oatmeal was sprinkled on or near it, with especial care at first that the grains were spread neither too widely nor too thickly lest contaminants overgrow the culture.

For the steps preliminary to purification, a piece of paper toweling bearing the plasmodium was placed on a sterile agar plate to which the myxomycete soon migrated. Transfers were made from the agar to the media used in purification, and the rest of the plasmodium saved as a sclerotium by slow drying (16).

PURIFICATION METHODS.—In the first or migration method small pieces (approximately  $2 \times 1$  mm.) of a plasmodium prepared as previously described were cut out, together with the underlying agar, and each placed in a 6.0 WA plate 1 cm. from the periphery. After the plasmodium had migrated over the plate as far as it could without crossing its own track, it was inoculated into a second similar plate and a small fragment inoculated simultaneously into a YED tube.

Three successive transfers of the myxomycete were generally sufficient to free a plasmodium of contaminants, and at each transfer a portion was placed in a YED tube which was incubated for 5 days at  $25^{\circ}$  C. before judgment was made on the purity of the plasmodium. Only rarely did a tube remaining clear for 5 days become cloudy later.

A pure plasmodium was inoculated into OA flasks simultaneously with the organism desired for two-membered culture, and into 6.0 WA slants streaked with YA for pure culture.

In the second, or enrichment culture method, each 6.0 WA plate was streaked along a diameter to a length of 5 cm. with a suspension of *S. ellipsoideus* (YL), the plasmodium placed at one end, and the plates examined daily for migration and feeding. After reaching the end of the streak the plasmodium was inoculated into a second similar plate and small fragments inoculated both into YE and YED tubes, which were incubated as described for the migration method. Both broths were used here, for although the YED is the more general culture medium, a heavy yeast growth often obscured slow-growing bacteria; YE served as a check since the yeast did not grow in it appreciably. On the other hand, a sugar-requiring contaminant would not grow in the YE and therefore might not be revealed.

After use of the enrichment method it was seldom necessary to inoculate *S. ellipsoideus* into two-membered cultures as the myxomycete usually carries sufficient yeast with it. But for pure cultures the plasmodium is desired without the yeast, and the *Saccharomyces* was removed by one or two migrations over non-nutrient agar.

PURE AND TWO-MEMBERED CULTURE.—Two-membered cultures were grown as described, in 125-ml. OA flasks into which the yeast and the plasmodium were inoculated simultaneously. Such cultures may thrive for a month or more without the need of transferring, but care must be taken that plasmodia are transferred before they decline too far in vigor, otherwise they may die overnight, presumably from the effects of accumulated wastes.

Attempts at using media other than oatmeal for cultures have not proved satisfactory, usually because these media, Pabulum agar, YD agar, malt agar, and oatmeal mash, were richer and allowed too heavy a growth of the yeast.

Two-membered cultures have been attempted with organisms other than *Saccharomyces ellipsoideus*, such as *Torula aclotiana* (fig. 4A), the mycelia of *Alternaria* sp. and *Coprinus* sp., and various bacteria, but generally such cultures have not proved satisfactory.

For pure culture, slants of 6.0 UA were streaked with the autoclaved yeast in a manner exactly like that used for streaking living yeast. With the rough calibrations on the pipette as a guide, about 0.1 cc. YA was streaked the entire length of each slant and an uncontaminated plasmodium inoculated near the bottom.

Pure cultures were usually incubated at 25° C. to increase the rate of metabolism and hence the number of serial transfers in a given time. All other cultures were kept at room temperature (22° C.), although gross, two-membered, and pure cultures thrive between the temperatures of 12° and 28°, the limits used by the writer.

STORAGE OF CULTURES.—Gross cultures were sclerotized by the method of HOWARD (16) and kept in envelopes in the refrigerator. It is not yet known whether refrigeration adds to the length of time sclerotia may be kept in viable condition.

No altogether satisfactory means of storing two-membered and pure cultures could be devised. Pure cultures were allowed to sclero-

tize, while two-membered cultures were placed in the refrigerator at 6° C.

Pure culture sclerotia of *Badhamia foliicola*, the myxomycete most frequently used in these investigations, seem to remain viable for hardly more than a month. Only one out of about twenty-five germinated after 3 months, although most of the others were apparently

TABLE 1  
PLASMODIA IN PURE CULTURE

PLASMODIUM	STRAIN NO.	NO. OF PAS-SAGES IN CULTURE	LOCALITY AND DATE COLLECTED
<i>Badhamia foliicola</i> List.....	13	15	Stanford University, California; oak leaves; April, 1936
<i>Didymium squamulosum</i> Fr.....	14	9	Stanford University; bedding straw (as fruit); December, 1936
<i>Fuligo septica</i> Web..	15	14	Bakersfield, California; old willow logs; July, 1936
<i>Stemonitis axifera</i> MacBr.....	21	3	Stanford University; preying on <i>Auricularia</i> sp.; December, 1936
<i>Badhamia utricularis</i> Berk.....	22	4	Stanford University; preying on bracket fungus; January, 1937
<i>Physarum polycephalum</i> Schw.....	24	6	Howard's original isolate, through courtesy Dr. William Seifriz
<i>Plasmodium</i> 51 (has not fruited).....	51	8	Cambridge, Mass.; preying on <i>Exidia</i> on dead elm branch; April, 1938

normal under the microscope and did not discharge their color on wetting—a property of dead or moribund plasmodia and sclerotia. The use of infusion agars of various natural substrates (hay, leaves, etc.) was unsuccessful, as were 0.02 molar concentrations of the excystment substances citrate and malate (38). Sclerotia of two-membered and gross cultures of the same species are viable for a longer period, gross culture sclerotia having been brought into a vegetative state after 2 years of dormancy.

The species listed in table 1 have been obtained in pure and two-membered cultures by one or both of these methods.

## Results

### PURIFICATION EXPERIMENTS

It is apparent that migration over non-nutrient agar might eventually free a plasmodium of contaminants by simple attrition and by the washing of the protoplasmic surface by the constantly secreted slime. Indeed it has been observed that plasmodia with an adherent tenacious slime (*Fuligo*, *Didymium*) are less easily obtained pure by migration than are those with a very loose, abundant slime (*Physarum*, *Badhamia foliicola*). It was therefore surprising that the first attempts at obtaining uncontaminated plasmodia by migration gave completely negative results, so the method was abandoned in favor of enrichment.

An enrichment culture meets the minimum requirements of the organism desired in mass culture, but at the same time it does not satisfy the needs of the associated undesired organisms. Living, washed yeast cells can serve as food for only those organisms which can engulf them, and therefore are admirable for plasmodial enrichment cultures.

For the early specific enrichment cultures several yeasts were employed. Of these, *Saccharomyces ellipsoideus* Hansen, 8.2.1. C.B.S.; *S. cerevisae* Hansen, 8.1.6. C.B.S.; and FF-17 Department of Bacteriology, Stanford University, gave the most promising results. The strain of *S. ellipsoideus* was found to be the most useful yeast as it is easily grown in liquid culture under not too critical conditions, is quickly eliminated by plasmodia crawling over plain agar as shown in figure 2B, and in addition could be used to grow all plasmodia on enrichment plates (with the exception of *Physarum polycephalum*) which could be grown on oatmeal agar. The *S. cerevisae* strains were almost equally useful, but being bottom yeasts their tendency to clump together and settle out quickly hindered even streaking.

It may be noted that although other yeasts used<sup>5</sup> often were

<sup>5</sup> *Debaryomyces hudeloi* da Fonseca, M-182; *Nectaromyces alpinus* Kluyver, M-1; *Willia anomala* Hansen, M-63; *Zygosaccharomyces pastori* Guill., M-174; all of the Harvard Medical Collection, and *Sporobolomyces salmonicolor* Kluyver & van Niel, 28.1.1. C.B.S.; *Torula aclotiana* Kufferath, 15.16.1 C.B.S.; *T. dattila* Kluyver, 15.1.1. C.B.S.; *T. monosa* Kluyver, 15.2.1. C.B.S.

favorable for growth of the plasmodium, they were impracticable because they were slow-growing in culture, or were small, glutinous, and therefore very difficult to remove from the plasmodium—disadvantages lacking in *S. ellipsoideus*.

After the plasmodia have been shown to be free of contaminating organisms, enrichment can often be carried a step further to rid the myxomycete of its yeast; all that is required is a sugar-free medium which supports the growth of myxomycetes. Such a medium is sugar-free autoclaved yeast. But since most commercial brands of bakers' yeast contain appreciable amounts of sugar after autoclaving, it has been found preferable to allow plasmodia from enrichment plates to migrate over one or two plates of pH 6.0 washed agar before placing them on autoclaved yeast slants which contain enough sugar to allow the growth of *S. ellipsoideus*.

At first considerable success was obtained with the enrichment method, but later plasmodia (*Fuligo septica*, *Didymium squamulosum*) were obtained in the field which resisted nearly all attempts to free them of contaminants. These plasmodia fed well but slowly on the yeast, and often arrived at the end of streaks more visibly contaminated than when they were inoculated. Sometimes a veritable garden of pseudomonads and bacilli could be observed at the advancing plasmodial edge under the microscope. From those amenable to the enrichment method these plasmodia differed in forming long straggling masses often extending back to the point of inoculation, and in their slow feeding especially (fig. 1A, B). Under these conditions the yeast, always in a state of inanition on washed agar plates, died and autolyzed, thereby furnishing nutrient for the contaminants and destroying the conditions of enrichment. Yeast cells removed from these streaks and examined in weak aqueous methylene blue, which stains only dead cells, showed many cells, even up to half in the field, staining with the dye, while yeast removed from a culture of a relatively fast-moving plasmodium showed very few cells taking the stain after the plasmodia had crawled comparable distances.

These plasmodia so refractory to the enrichment method were obtained in pure culture by a modification of the formerly unsuccessful migration method. In the earlier method, large (with a fan 0.5 cm.

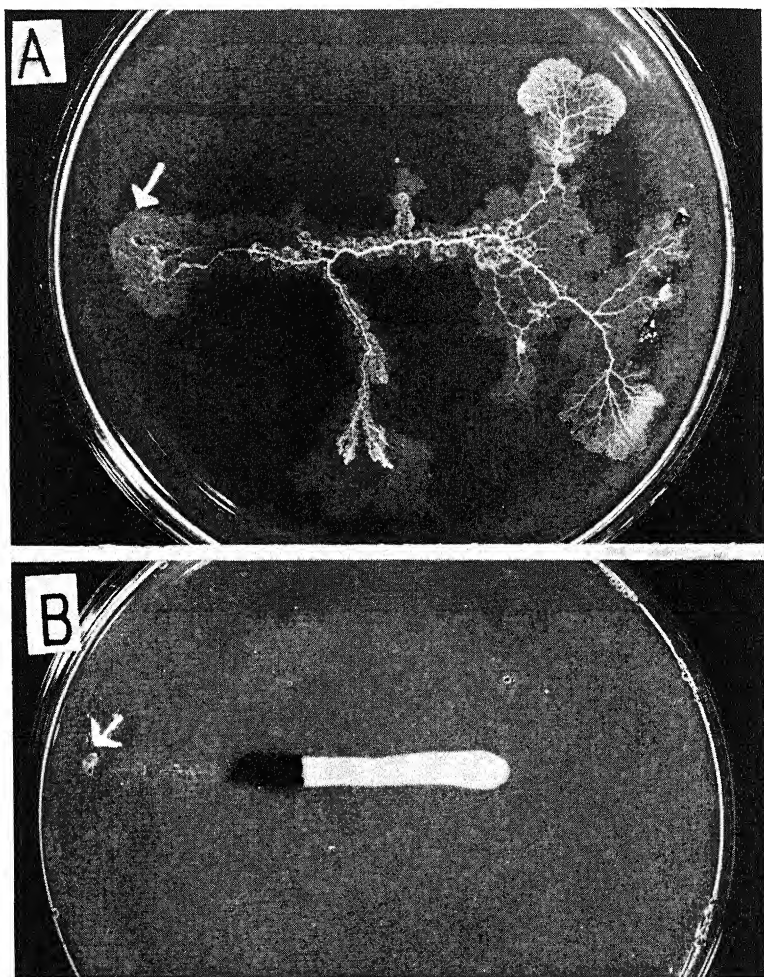


FIG. 1.—Types of feeding exhibited by plasmodia on living yeast enrichment plates (YL, 6.0 WA). *A*: *Fuligo septica*, slow-feeding plasmodium with diffuse fan. *B*: *Badhamia foliicola*, fast-feeding plasmodium with compact fan. With orthochromatic plates these bright orange-yellow plasmodia photograph as black (*cf.* fig. 4*A* and compare fig. 4*B*, taken with panchromatic plate). White streak extending to right of plasmodium is the yeast not yet consumed. Arrows in *A* and *B* point to sites of inoculation.

diameter or larger) plasmodia were allowed to migrate, while in the later successful method smaller plasmodia were employed.

The impression that the rate of feeding determined comparative usefulness of the enrichment and migration plate methods was checked more critically by measuring the rates of crawling on both kinds of plates. At the same time the possible influence of pH on crawling rates was observed.

In these studies washed agar plates at pH 5.5 and at pH 6.5 were prepared and half the plates at each pH streaked with a suspension of YL for a length of 5 cm. along the diameter of each plate. Fragments from contaminated plasmodia were placed on each migration and enrichment plate and the cultures incubated in the dark at room temperature (22° C.). For uniformity, the inocula were of as nearly the same size as possible and were taken from similar regions of each plasmodium. Because of the diminution of size on inanition, the inoculations for the migration plates were larger (0.5 cm.<sup>2</sup>) than those for the enrichment plates (2 mm.<sup>2</sup>). Plates were examined daily and distances migrated on both kinds of plates noted. After a migration of 5 cm., plasmodia were transferred to corresponding plates and at the same time inoculated into broths. Three such serial plates were used for each culture.

Each migration rate recorded in table 2 is an average of eight plasmodia of the same clone. Migration rates of the starred (\*) plasmodia included in the table were measured previous to the rates of the others. Hence the data though obtained under similar conditions are not strictly comparable, but are included because of their interest.

It is seen from table 2 that for the enrichment method to be feasible, plasmodia must crawl up the streak at a minimum rate of 1.2 cm. per 24 hours. A comparison of the number started, number pure, and number of plasmodia remaining at the conclusion of the experiment shows that not all plates in a series could be carried through to completion. Thus *Fuligo septica*, plasmodium 52, often sclerotized on the migration plates and could not be revived, a tendency not exhibited to so marked a degree by other strains of this species.

The extremely fast-moving plasmodia *Badhamia foliicola* and

*Physarum polycephalum* were in nearly all cases pure at the end of the first passage on the migration plates. *P. polycephalum* spread so rapidly that its rate could not be measured by daily inspection, and therefore it is indicated only as more than 5 cm. per day.

TABLE 2

PLASMODIUM	PH OF AGAR	MIGRATION OVER YEAST STREAKS				MIGRATION OVER PLAIN AGAR			
		DAILY CRAWLING RATE (CM.)	No. OF CASES STARTED	TO-TAL PURE AT END	TO-TAL AT END	DAILY CRAWLING RATE (CM.)	No. OF CASES STARTED	TO-TAL PURE AT END	TOTAL AT END
Badhamia foliicola.....	5.5	1.4	8	8	8	6.2	8	8	8} (1st pas-sage)
	6.5	1.2	8	8	8	6.8	8	7	
Physarum polycephalum....	5.5	No feeding in any case			.....	5+	8	8	8} (1st pas-sage)
	6.5	No feeding in any case			.....	5+	8	8	
Stemonitis axifera.....	5.5	1.6	8	6	7	4.1	8	8	8 8
	6.5	1.8	8	3	4	4.8	8	8	
Plasmodium no. 51.....	5.5	1.7	8	5	6	2.3	8	1	2
	6.5	1.4	8	5	6	3.1	8	3	4
Fuligo septica no. 52.....	5.5	0.6	8	0	3	2.0	8	0	1
	6.5	0.6	8	0	5	1.9	8	1	2
Fuligo septica* no. 15.....	5.0-7.0	1.6	20	9	19	1.5	20	19	20
Didymium squamulosum*....	6.0	1.0	6	0	6	.....	6	6	6

\* See statement in text.

The behavior of *Fuligo septica* (plasmodium 15) in this particular experiment is an apparent exception to the slow-moving *Fuligo* spp. Neither before nor after this experiment has any success been obtained with the enrichment method with plasmodium 15 nor any other *Fuligo*.

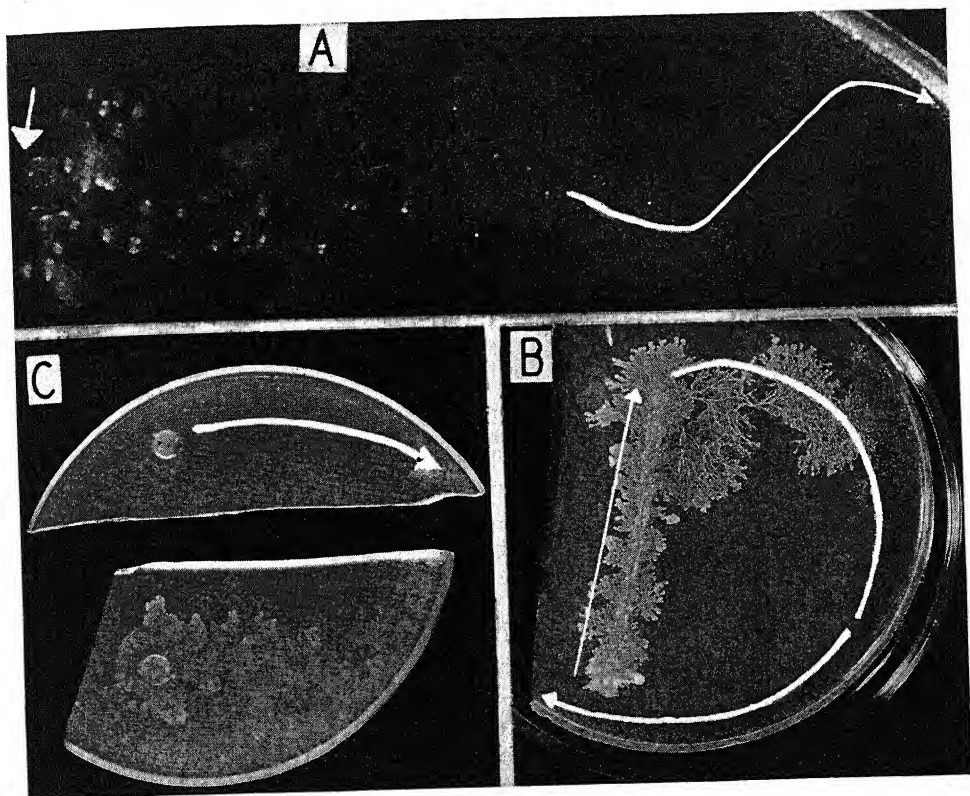


FIG. 2.—Growth of micro-organisms left in situ by plasmodia which in all cases have been removed and their paths indicated by long curved arrows. In *A* and *B*, agar was placed on surface of nutrient plates; the nutrient diffusing upward allowed growth of the deposited micro-organisms into colonies. *A*: migration plate showing rapid loss of bacteria indicated by thinning out of colonies with increasing distance from inoculum (indicated by short arrow). *B*: enrichment plate showing speedy loss of *S. ellipsoideus*. Slender arrow indicates extent of yeast streak. Break in heavy arrow shows last yeast colony developing. Note extent to which plasmodium has spread the yeast to form an image of itself. *C*: differences in nutrient content of washed and unwashed agars. Upper portion, from washed-agar migration plate, shows no development of bacteria in track of plasmodium. Lower portion, from unwashed-agar plate, shows development of bacteria without addition of nutrient.

It is also seen from table 2 that there was little difference in the obtained results ascribable to the influence of the hydrogen ion concentrations in the range employed. The agar becomes increasingly soft and difficult to work with as the pH decreases, but on the other hand, agars of pH's higher than 6.5 become increasingly unfavorable to the vigor of the myxomycete. Agar of pH 6.0 lacks these disadvantages and was therefore employed in all subsequent work.

The migration method, besides being simpler, gives apparently better results on the whole than does the enrichment method. This superiority is only apparent, for it has too often happened that a plasmodium has been so completely devitalized by continued inanition that it could not be transferred successfully to two-membered or pure culture. The enrichment method when feasible always insures an abundance of plasmodium with which to work; there is no danger of sclerotization, and little danger of the plasmodium crossing its own track and picking up contaminants it had left behind.

The freeing from bacteria was illustrated more qualitatively and picturesquely by cutting out the non-nutrient agar from an enrichment or migration plate over which a plasmodium had crawled and placing it on a yeast-extract-dextrose agar plate. The nutrient diffused up through the plain agar and allowed development of colonies from the deposited micro-organisms *in situ* (fig. 2A). The slowly moving plasmodia do not rid themselves very speedily of bacteria, and indeed the number of bacteria on the agar may be so great that the colonies coalesce.

By the same means the rapid loss of *S. ellipsoideus* could be demonstrated as shown in figure 2B, in which the last visible colony developed when the myxomycete had migrated about 7 cm. after leaving the end of the depleted yeast streak.

#### ADAPTATION PHENOMENA

It is well known among protozoologists and bacteriologists (20, p. 162 *et seq.*) that organisms of a species transferred to a new medium may show extremely wide variation in growth in parallel cultures, and also that organisms may be trained to grow on a medium previously unsuitable. A similar phenomenon has been observed by the writer in the culture of myxomycetes.

In the course of these experiments it was found advantageous to make several parallel inoculations of a newly purified plasmodium into the slants of autoclaved yeast. Of several such cultures started, often only a few began development, and from the most robust of these (fig. 3B, C) the myxomycete was serially subcultured. Alternatively, fewer tubes were used and a very large inoculum placed in each (as in BRAUN and CAHN-BRONNER's technique for the adaptive nutrition of bacteria, KNIGHT *loc. cit.*), but this mass-inoculation method is technically inferior, for the smaller the inoculum in a successful subculture on a new medium the more assurance there is that the medium is complete (13).

From the few experiments so far carried out, three adaptive phenomena may be recognized:

1. A plasmodium may be trained to grow under conditions which are usually inimical. For example, *Badhamia foliicola* was made to grow at a pH of 7.4 by both direct mass inoculation from a milieu of pH 6.0 and by using small inoculations and gradually raising the pH at each transfer. Small inoculations made directly into an identical medium remained immobile and died.

2. A plasmodium may be trained to utilize an initially poor nutrient. For example, *Physarum polycephalum* refused to grow in two-membered culture on oatmeal with *S. ellipsoideus* and in pure culture on autoclaved yeast when first inoculated in the usual manner. By the use of a number of inoculations in the first case, and of mass inoculation in the second, successful cultures were obtained. The writer has observed a higher degree of variation of behavior with *P. polycephalum* than with any other plasmodium.

3. A plasmodium regularly increases in vigor of growth with serial transfer on a new medium, and often within the first transfer (fig. 3A). This increasingly efficient utilization of the medium is not due to the size of the inoculum, for equally small inocula take up the yeast completely from the first on subculture. With the exception of the *Fuligo* spp., the writer's plasmodia all show OEHLER's (26) *wallartig* instead of his *zerstreute Wachstum*.

Since all the plasmodia studied showed one or more of these adaptation phenomena, it seemed doubtful at first whether controlled nutritional work could ever be done with such variable or-

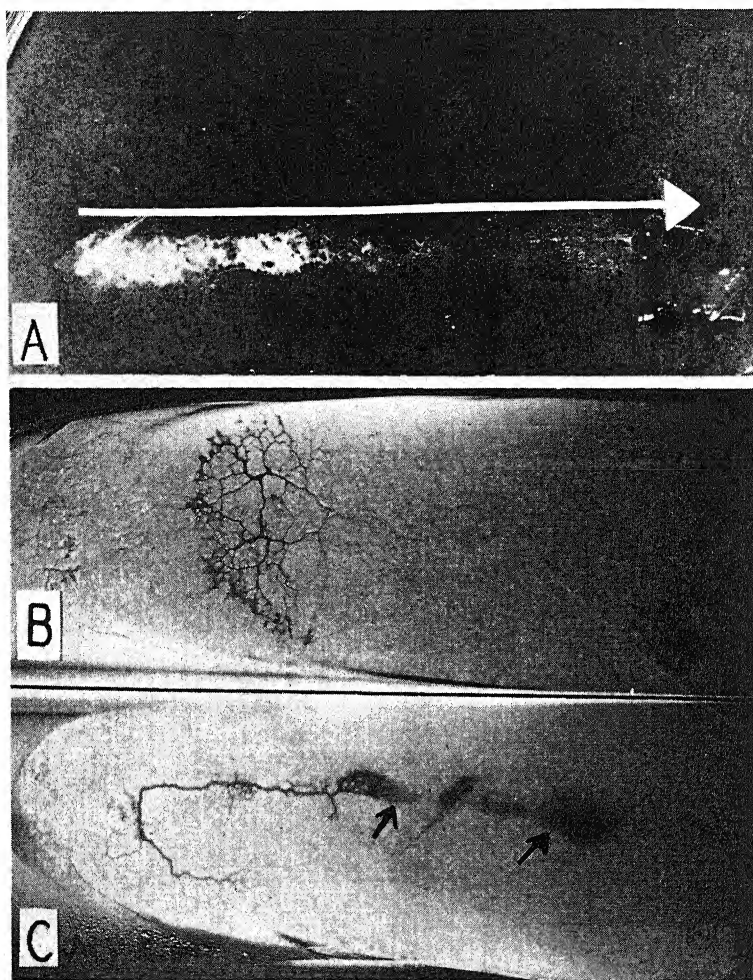


FIG. 3.—Adaptation phenomena exhibited by myxomycete plasmodia. *A*: increased utilization of *S. ellipsoideus* by plasmodium (plasmodium 51) on enrichment plate. Plasmodium inoculated at point indicated by tail of arrow leaves decreasing amounts of yeast behind as it migrates to position indicated by arrowhead. *B*, *C*: plasmodia of *Badhamia foliicola* from same clone in which differences of vigor in growth exhibited by original isolates have persisted through second transfer. *B*: second transfer on auto-claved yeast from isolate showing good growth on this medium. All yeast has been utilized and plasmodium is now in state of inanition. *C*: second transfer from isolate showing poor growth; same time interval as for *B*. Note large masses of untouched yeast (indicated by arrows).

ganisms; but continued cultures have produced laboratory strains which are highly consistent in their behavior under similar conditions.

#### FACTORS INFLUENCING CULTURE

The inadvisability of a rich medium which allows excessive growth of the second member has already been mentioned in connection with two-membered cultures. In pure culture work it was soon observed that too much autoclaved yeast was inimical or even fatal to growth. There seemed to be two possible ways of interpreting this phenomenon: (a) too much dissolved organic material was present; (b) too heavy a layer of yeast physically hindered feeding of the plasmodia. Experiments undertaken with *Badhamia foliicola* indicated physical hindrance to be the factor in this instance.

In the experiments, two sets of autoclaved yeast were prepared. In both the yeast was autoclaved in the usual manner, but in one set soluble substances were washed away by repeated centrifuging in distilled water and the yeast re-autoclaved after adjusting the suspension to initial cell density, while the second set was merely re-autoclaved. The following series of media were prepared:

1. Slants containing 8 cc. of 2 per cent unwashed agar, pH 6.0, containing 1 cc. of the supernatant fluid of autoclaved yeast, and each streaked with 0.1 cc. of a suspension of washed, re-autoclaved yeast cells.
2. Slants containing 8 cc. of agar prepared as before, but each streaked with 0.1 cc. of a suspension of unwashed, re-autoclaved yeast cells.
3. Slants containing 8 cc. of 6.0 unwashed agar each streaked with 1 cc. washed, re-autoclaved suspension.
4. Slants containing 8 cc. of 6.0 unwashed agar each streaked with 1 cc. unwashed, re-autoclaved yeast suspension.

Only series 1 and 2 gave good growth, and therefore it seems clear that it is the quantity of yeast cells on the surface rather than the soluble substances which are inhibitory.

At one stage of the research, maintenance of all myxomycetes in culture was attempted on washed agar in order to reduce the number of separate media and the number of unknown factors contained in

unwashed agar. These cultures without exception decreased in vigor on passage until finally some gave no perceptible growth after transfer. It seemed obvious that some necessary substances, probably minerals, had been leached out by the washing process; and as plasmodia grew well in enrichment cultures on washed agar, these presumable substances must be in intact yeast cells and in unwashed agar. That unknown minerals are necessary was shown by the following experiment.

The ash of unwashed agar was added to washed agar (2 per cent) adjusted to a final pH of 6.0, slanted, and streaked with autoclaved yeast. Controls of unwashed agar and washed agar without minerals were run, and *Badhamia foliicola* was used as the test myxomycete because of its sensitivity to the washing process. Growth on the washed agar was much less than on both the unwashed agar and the washed-agar-plus-agar-ash, little if any difference between the latter two being noticeable.

#### ATTEMPTED PURE CULTURE ON OTHER MEDIA

Various investigators have used a number of media for culture of the myxomycetes. In most instances the culture was admittedly or obviously gross, and need be discussed no further here (DEBARY, LISTER, KLEBS, CONSTANTINEANU, MILLER, BELAR, *et al.*).

Early in the present work it was expected that myxomycetes would grow well on pure culture on oatmeal agar (16), and that they would also grow on other common vegetable substrata. Actually no such growth took place when a few flasks and tubes of oatmeal agar were inoculated with *Physarum polycephalum* freed of contaminants. In a similar series of experiments with *Badhamia foliicola*, plugs of autoclaved potato, carrot, banana, and apple were also employed with negative results. No growth of either plasmodium was found in attempted pure culture on the following autoclaved media: yeast extract agar, yeast extract-dextrose agar, malt agar, oatmeal agar, oatmeal mash, pea mash, pea agar, pea infusion agar, *Agaricus campestris* sporophores, and *A. campestris* sporophore agar. That some of these media were not particularly inimical to development was shown by the growth of plasmodia in accidentally contaminated

vessels. In short, wherever bacterial contamination occurred, growth took place.

Because of the importance which oatmeal has achieved as a substrate for the cultivation of plasmodia (15, 16, 7) and the belief that it is a pure culture and a "synthetic" medium (36), it was more thoroughly investigated as a possible medium for pure culture and as a medium for two-membered culture.

In the earlier experiments three factors besides the lack of microorganisms may have been responsible for the failure of the plasmodia to grow: (a) the oatmeal grains imbedded in the agar may have been physically unavailable; (b) the inocula may have been too small; and (c) the hydrogen ion concentration may have been unfavorable.

To settle the first and third points, oatmeal agar of pH 6.0 (a pH known to be favorable) was prepared in the ordinary way, and for comparison, flasks of 6.0 unwashed agar were sprinkled with sterile oatmeal grains after the agar had solidified. HOWARD (15) mentions the difficulty of freeing *Physarum polycephalum* from a small toruloid yeast. Using this fact as a clue, two-membered cultures of *Torula acclotiana* 15.16.1 C.B.S. and *P. polycephalum* were made on both the oatmeal agar and the sterile oatmeal grains. Large (even to an inoculum covering half the surface of the flask) (cf. fig. 4C) and small inoculations were made into the two media, each with and without the *Torula*. Abundant growth was obtained on both media wherever the *Torula* was present, while no growth was perceptible in its absence (fig. 4). It may be mentioned that *P. polycephalum* has shown consistently better growth on *T. acclotiana* than on any other organism in two-membered culture on the same supplied substrate.

This problem was investigated further. *Physarum polycephalum* and *Badhamia foliicola* were grown in gross culture for one week on oatmeal at the slightly low hydrogen ion concentration of pH 7.0 to favor growth of bacteria. The bacteria were isolated by streaking on oatmeal decoction agar and pure culture isolations made from the different colonies.

Two-membered cultures were prepared by inoculating pure culture plasmodia into the oatmeal agar and "oatmeal-grain" flasks and placing a few drops of a suspension of each bacterium isolated directly on the plasmodial inoculum. Controls were (a) pure culture

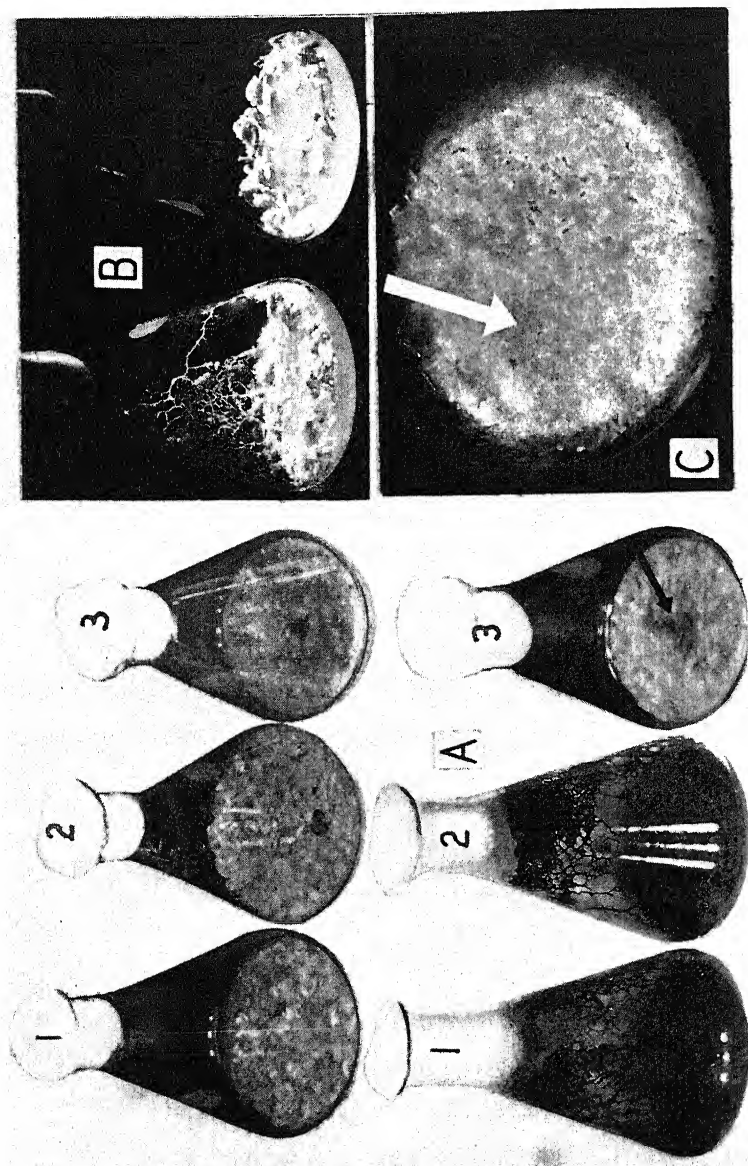


FIG. 4.—Two-membered and attempted pure culture on oatmeal. A: plasmodia of *Badhamia foliicola* on oatmeal agar, left to right (both rows): 1, two-membered culture with *Torula acclotiana*; 2, same with *Flavobacterium ceramicola*; 3, attempted pure culture on oatmeal. Upper row, 12 hours after inoculation; lower row, 2 weeks later. Arrow in 3 points to dead inoculum in attempted pure culture flask. B: *Physarum polycephalum* with sterile oatmeal on agar. Left: two-membered culture with *T. acclotiana* showing excellent development of plasmodium. Right: attempted pure culture showing complete absence of growth. C: large inoculum of *P. polycephalum*; agar on which plasmodium was carried into flask covers left half of oatmeal agar surface. Plasmodium eroded oatmeal agar at right of inoculum; in absence of second member it crawled back to original substrate and died after no perceptible growth. Arrow points to disintegrated plasmodium.

plasmodia inoculated into the flasks without bacteria, and (b) plasmodia inoculated into flasks with suspensions of scraping from gross cultures.

In most of the flasks inoculated with bacteria there was development, often to so great a degree that the agar was riddled with plasmodial strands, the walls of the flasks covered, and even the cotton plugs penetrated by the plasmodium (fig. 4A). In those flasks which contained no demonstrable living organism other than the plasmodia there was little or no development, the plasmodia crawling about sluggishly, eroding the grains only slightly, and finally breaking up into small pulvinate masses which died and discharged their color into the substrate. The controls inoculated with the mixture of organisms from gross culture showed some development, and *Physarum polycephalum* fruited in several of the control flasks; but generally the rank growth of molds, yeasts, and various bacteria inhibited the further development of plasmodia from reaching a degree commensurate with that of the most favorable two-membered cultures.

In those few two-membered cultures in which growth did not take place it could be shown that the bacteria raised the pH to an unfavorable degree, grew excessively fast, or formed slimy, mucilaginous colonies difficult to attack.<sup>6</sup>

Attempts have been made to obtain pure culture on oatmeal agar with the other myxomycetes in stock, but the results have been essentially the same as in the cases of *Physarum polycephalum* and *Badhamia foliicola*; namely, sluggish crawling, slight erosion of the grains, breaking up into small pulvinate masses, and finally death, after little or no perceptible increase in size of the plasmodium during its life on the oatmeal. *Fuligo septica*, to be sure, did show a very slight growth, but it too died within 3 weeks; in contrast this organism has been carried without transfer in regular two-membered culture for over 6 weeks at room temperature and over 3 months at 6°.

In these experiments no essential difference was found in the use of very small inocula and of those of 0.5 cm. or more in fan diameter.

<sup>6</sup> The relationships existing in two-membered cultures and the erosion of oatmeal grains even by pure culture plasmodia are to be taken up in a succeeding paper.

The smaller inocula took longer to grow to the same final quantity as the larger ones, and the conditions were somewhat more critical, as a fast-growing second member could overrun and stale the culture before the myxomycete became sufficiently established to keep it in check.

### Discussion

#### TYPE OF NUTRITION

That the plasmodia are not in competition with the yeast for the supplied medium, as CHRZĄSZCZ (10) stated, is shown by the experiments in which yeast alone supplied sufficient nourishment, and in its absence on a given medium there was no growth. Nor is there any indication that the secreted slime or metabolic products of the yeast are the important nutrients, as suggested by HENNEBERG (14). On the contrary the yeast cells are engulfed and destroyed, as microscopic examination of a yeast streak over which a plasmodium has crawled and fed reveals only a very few scattered cells instead of a densely packed, opaque yeast mass, or even the empty cell walls of such a mass.

#### PURE CULTURE

SKUPIENSKI (34), VON STOSCH (37), and PINOY (28, 29) declare it impossible to grow myxomycete plasmodia in pure culture, while HOWARD (15) and SKUPIENSKI (32) claim pure culture on media similar to those unsuccessfully employed by the writer (oatmeal agar, carrot agar, hay agar, and agaric agar).

These discrepancies may be explicable in the light of the present investigations. For example, it has previously been shown that for some plasmodia a number of transfers must be made to a pure culture medium before a few of the inoculations are successful. This was found to be particularly true of *Didymium squamulosum*, *Phy-sarum polycephalum*, *Stemonitis axifera*, and *Fuligo septica*. So great is the variation with the freshly isolated *Didymium* that at present the writer is not certain that it can be carried through indefinitely in pure culture.

Other factors have been discussed, such as size of inoculum, amount of minerals present, and concentration of nutrients in the projected pure culture medium. In view of these complicating fac-

tors, it is perhaps not surprising that SKUPIENSKI (34) and VON STOSCH obtained negative results. The hydrogen ion concentration, although not very critical, may also have been left out of account.

SKUPIENSKI (32) claimed pure culture of *Didymium nigripes* on carrot infusion agar. HOWARD (15, 16) indicates the same results for *Physarum polycephalum* on oatmeal agar and sterilized fungus sporophores. The only criterion of purity recorded in the French résumé of SKUPIENSKI's paper and by HOWARD (personal communication) is the clarity of the surface of the agar.

NADSON (25) long ago pointed out the unreliability of such a procedure, and VOUK (39) subsequently working with *D. nigripes*, the same species used by SKUPIENSKI, showed that plasmodia seldom leave well defined colonies in their wake but that the bacteria are distributed along the track and around the periphery of the plasmodium.

In consideration of the manner in which a plasmodium sweeps over a surface, it is difficult to expect contaminants to form defined colonies, and it is well known among microbiologists that the plating method requires a dry and undisturbed surface, especially in the case of very motile pseudomonads and those bacteria which form fluid mucilaginous colonies. Almost all the twenty-five bacteria isolated from the six different plasmodia produce on agar peculiarly nondescript and spreading colonies of a white, buff, or yellow color, while some colonies are practically transparent. In fact all the bacteria occurring commonly in the streak plates, and presumably the most abundant species in the gross cultures, could be so characterized.

When we add to the inherent difficulties just mentioned the opaque, heterogeneous appearance and light buff color of oatmeal agar, its low protein content, and the usually high acidity of such vegetable media, it is not surprising that bacteria which form such nondescript colonies should be difficult to detect macroscopically. When in addition bacterial colonies are broken up and spread either artificially or by the divagations of a restless plasmodium, only careful microscopical and cultural examination can be expected to reveal the presence of the contaminants. For example, uninoculated flasks have been compared with those inoculated over the entire surface

with an extremely actively growing pseudomonad common in all oatmeal gross cultures examined, and during the period of most active development only a slightly moister-appearing surface of the inoculated flasks (as if they were fresh from the autoclave) as compared with the controls indicated the presence of the pseudomonad, although a suspension prepared from the inoculated flask showed a field crowded with the actively motile bacteria. As a matter of fact even in vigorous two-membered culture, the ordinarily very visible and defined colonies of *Torula aclotiana* and *Saccharomyces ellipsoideus* are often spread out and overrun by the plasmodium to such a degree that microscopic examination is necessary to demonstrate the yeast.

It is pertinent at this point to quote from OEHLER's paper on a parallel research and discussion of protozoan cultures:

"Wie bei allen Bakterien-Amöbenzuchtversuchen, so ist auch bei der Amöbenzucht auf abgetöteten Bakterien ständige Prüfung auf Reinheit und Sterilität bei jedem einzelnen Versuchsakte nötig. Wenn man nicht immer und immer wieder alle Versuchsglieder auf Reinheit und Sterilität nachprüft, kann man das Wunderbarste finden" (26, p. 185).

### Summary

1. The literature on the nutrition of myxomycete plasmodia is discussed critically from the standpoints of ingestion, two-membered culture (a special case of association culture with two different organisms), and pure culture. It is shown that the reports of pure culture are based on misconceptions or are at least open to criticism.

2. Techniques are described whereby myxomycete plasmodia may be obtained free of contaminants and subsequently grown in two-membered culture with *Saccharomyces ellipsoideus*, or some other micro-organism, and in pure culture on autoclaved yeast. Plasmodia may utilize living or autoclaved yeast as the sole source of nutrient.

3. The behavior of plasmodia in culture is discussed, and it is shown that myxomycete plasmodia may exhibit adaptation to cultural conditions.

4. Attempted pure culture on oatmeal and similar vegetable media was unsuccessful, in contradiction to reported pure culture on these media.

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# USE OF THE FEULGEN REACTION IN CYTOLOGY

## I. EFFECT OF FIXATIVES ON THE REACTION

B. B. HILLARY

(WITH THREE FIGURES)

### Introduction

The Feulgen reaction was developed in 1924 by FEULGEN and ROSSENBECK (11) as a microchemical test for thymonucleic acid. This reaction was primarily intended to distinguish between the type of nucleic acid (thymonucleic) considered to contain a hexose radical and other similar acids containing pentose radicals. At the time FEULGEN carried out his experiments the exact nature of the carbohydrate complex of the thymonucleic acid molecule was not understood. Since then the investigations of LEVENE, MIKESKA, and MORI (17) have shown that the carbohydrate is not a hexose but a pentose. Notwithstanding this, the reasoning of FEULGEN and his collaborators regarding the theory of the reaction still holds.

The Feulgen reaction is carried out in two steps. The first is mild acid hydrolysis, which breaks the binding between the purine bases and the carbohydrate complex of the nucleic acid. This splitting off of the purines frees the aldehyde groups of the aldo-pentose sugars. The second step involves a chemical reaction between the liberated aldehyde groups and leuco basic fuchsin (fuchsin sulphurous acid). According to FEULGEN, this results in the synthesis of a new dye compound, which is violet in color as compared with the red of the basic dye.

It was formerly considered that there was a difference between the nucleic acid of plant and animal nuclei. Recently FEULGEN, BEHRENS, and MAHDIHASSAN (10) have developed a method of separating the nuclei of wheat germs from other parts of the cells and thereby extracting a pure plant nucleic acid from the nuclei. This nucleic acid was found to contain exactly the same groups as thymonucleic acid. Thus plant and animal nucleic acids are one and the same thing and the nucleal reaction works equally well with both.

FEULGEN (8) has demonstrated the value of the nucleal stain in both plant and animal materials. He has shown the great selectivity and specificity of the stain, in that it stains only the chromatin parts of the nucleus and leaves unstained the nucleolus as well as all parts of the cell outside the nucleus. This makes it very valuable for cytological work, as it stains the chromosomes with remarkable sharpness and clearness. Unlike most other cytological stains it does not require differentiation to make the chromosomes distinguishable; therefore it provides a much more certain and usually a much clearer picture of the finer details of chromosome structure.

In spite of these apparent advantages, adoption of the Feulgen nucleal stain by cytologists has been slow. With plants this is perhaps due to the fact that the reported differences between plant and animal nucleic acids made investigators feel that the reaction was uncertain in them. Another cause is the difficulty of obtaining a basic fuchsin suitable for use with the Feulgen reaction, as the impurities of the stain sometimes color the cytoplasm. The investigations of DE TOMASI (28) and SCANLAN and MELIN (23) have shown that this can be overcome by the use of a specially purified pararosanalin dye. COLEMAN (7) found a simple and more efficient method of overcoming this difficulty. By filtering the bleached solution with active charcoal, which removed the impurities, an absolutely colorless leuco basic fuchsin could be obtained from any sample of basic fuchsin.

Another retarding influence to the use of this stain was that FEULGEN recommended the use of a sublimate-acetic fixative, which is a poor cytological fixative. His reason for recommending this was that he considered fixatives containing oxidizing compounds such as chromic acid would be apt to destroy the nuclear material. Likewise he warned against the use of formalin because it is an aldehyde which would give the same color reaction. We now know that these compounds, at the concentrations used in fixatives, have no deleterious effect on the stain.

One of the greatest drawbacks to the use of this stain is the fact that there have been frequent and often incorrect reports of a negative stain reaction. For example, BOAS and BIECHELE (4) report a negative reaction in 138 out of 224 plants investigated. These results

cannot be accepted because the methods used were faulty. MILOVIDOV (20) claims that in many cases such results are due to the presence of tannins which inhibit penetration of reagents, but careful investigations which will be discussed later have shown that tannins do not normally inhibit the stain. *Spirogyra* was continually reported to give a negative reaction (15, 16, 20, 22, 26) until it was shown by GEITLER (12) that the stain was confined to widely separated minute chromocenters which had been overlooked by previous investigators. Such has been found to be the case in many forms.

In the extensive literature on the Feulgen reaction discussed by MILOVIDOV (21) there are only three papers that show a systematic analysis of the method and of the application of the nucleal stain and the difficulties encountered in its use. FEULGEN (8) outlined the general method and pointed out the extent and general limitations of its application. MILOVIDOV (20) analyzed the causes of a negative nucleal reaction and gave reasons for it, all of which are sound except for his conclusions regarding tannins. BAUER (1) investigated the effects of various fixatives and gave tables and graphs showing the time of hydrolysis that would give the best results with the various fixatives. Yet the recent literature is full of contradictions as to the effect of fixatives and time of hydrolysis on the reaction.

To try to clear up some of the confusions and contradictions regarding the nucleal stain, and to demonstrate its value in cytology, the following points were investigated.

a) The effect on the Feulgen reaction of various fixatives and of the different ingredients in these fixatives, thus extending the investigations of BAUER.

b) The effect of such substances as tannins, which have been suggested as interfering with the reaction.

c) The non-nucleal stains produced by leuco basic fuchsin, which may accompany and confuse the results of the nucleal stain.

d) The use of the nucleal stain in making clear such chromosome structures as the satellites and nuclear phases such as zygotene, diplotene, and metaphase stages, which cannot be studied in many species by ordinary methods of staining.

e) The use of the nucleal stain for the study of chromosome struc-

ture, and the conclusions that may be drawn as to the relation between structure and chemical composition.

f) The possibilities of the use of the nucleal stain to study the finer details of the structure of the chromonemata following proteolytic digestion; that is, the tryptic digestion method of CASPERS-SON (6).

g) The development of new techniques made possible by the Feulgen nucleal reaction.

The first three of these questions are dealt with in the present paper.

### Reagents

Reagents of the proper type are one of the prerequisites to success with the nucleal reaction, thus their preparation is important. The length of the period of hydrolysis is perhaps the most critical point of the reaction. The optimum of hydrolysis varies with the concentration of the acid and the temperature at which hydrolysis is carried out (9). The acid must therefore be of a standardized concentration and the temperature must be constant, since the procedures are based on these factors. Normal hydrochloric acid is used, and 60° C. has been adopted as a standard hydrolyzing temperature because it allows the optimum to be reached with a fairly short period of hydrolysis and it is not high enough to damage the structure of the nucleus.

The necessity of having a completely decolorized fuchsin sulphurous acid or leuco basic fuchsin has been mentioned. COLEMAN (7) gives the method of preparing such a solution. The SO<sub>2</sub> water, since it is not an integral part of the reaction but merely a bleaching rinse, need not be so carefully prepared as the other reagents. FEULGEN (8) gives the method of preparing this solution, but the use of potassium metabisulphite as suggested by DE TOMASI (28) produces a more satisfactory solution.

### Experimentation

Our present knowledge of the effects of fixing reagents on the Feulgen stain we owe almost exclusively to BAUER (1), who tested some twenty-four fixation mixtures in this connection. BAUER used chiefly animal cells (spermatocytes of grasshoppers), and showed

the effect of hydrolysis on the intensity of the stain with the various fixatives. While BAUER compared the results obtained by the nuclear stain with those obtained with a standard stain such as iron haematoxylin, he confined his tests in plants to sections of onion root tips, and of course did not attempt the use of the stain in the elucidation of chromosome structure. His attempts to account for the marked differential action of the various fixatives as affecting the time of hydrolysis do not seem very successful, while the method used (observation under the microscope) does not allow for a ready and rapid comparison of these effects. As he freely admits, the subjective element entered to no small degree into the construction of his graphs. It was felt therefore that BAUER's work should be reviewed and extended in an attempt to obtain a more objective picture of events, and at the same time to elucidate more fully the cause for the differential action of fixing reagents.

#### I. IN VITRO EXPERIMENTS

The in vitro experiments of FEULGEN were conducted in solutions of thymus nucleic acid in test tubes. Obviously such a method is out of the question where a fixing reagent is to be added, for it is impossible to separate it from the thymus nucleic acid solution after the fixing action has taken place. It was necessary therefore to include the thymus nucleic acid in a gel medium such as agar, which on cooling could be treated in a manner comparable with that used for plant tissue.

The agar blocks used in the in vitro experiments were made by adding 0.1 gm. of sodium nucleinate in 10 cc. of water to 100 cc. of boiling 5 per cent Bacto purified agar. This mixture was stirred for a few minutes, poured into plates, and after cooling and solidifying, cut into blocks. The blocks were actually cubes, with sides about 5 mm. in length, and the size was kept as uniform as possible so that accurate comparisons could be made. The agar blocks provide a convenient carrier for the nucleic acid as they can be passed through the various solutions without dissolution or leaching out of the acid. Also they permit macroscopical observation, as the vials containing the agar blocks can be placed side by side and whole series showing narrow differences in color can be compared at a

glance. In all experiments, the results of the agar blocks when compared with those obtained in cytological preparations (pollen mother cell smears of *Tradescantia virginiana*) were found to be identical.

The fixatives used represent four different types and belong to the four main basic groups of fixatives:

1. Feulgen's fixative (sublimate-acetic)
2. Carnoy's fluid (alcohol-acetic)
3. La Cour's 2 BE (chrom-osmic-acetic)
4. Belling's fixative (chrom-formol-acetic), a modification of the San Felice and Navashin type

RESULTS.—The first experiment with the agar blocks was to determine what effect the time of fixation had on the stain. Three series were run with each of the fixatives, the periods being 1, 24, and 48 hours, followed by 18 hours' washing with running water. In each series the periods of hydrolysis were 0, 1, 5, 10, 15, 20, 25, and 30 minutes at 60° C. with N HCl. The sample with no hydrolysis was used as a check to see whether any color was caused by some constituent of the fixative such as formalin. The agar blocks were left in leuco basic fuchsin for 30 minutes and allowed to bleach 24 hours in SO<sub>2</sub> water, during which period the solution was changed three times. In all three series no differences in the staining after a particular fixative could be observed. Thus the duration of fixation has no effect on the intensity of the stain. In the light of this, the results presented in this section are based on 24 hours' fixation and the preceding staining schedule.

The second experiment was to determine the optimum time of hydrolysis with the four fixatives. Along with this, a series of nucleic acid blocks with which no fixing agent had been used were treated in the same way, to serve as a check. The results of these experiments are shown in the graphs of figure 1. Agar blocks free from nucleic acid were also treated in the same manner to make sure that the colors produced were due to the added acid and not to any nucleic acid that might be in the agar itself.

The first type of curve (fig. 1A) is shown by the series with no fixation and by the Feulgen and Carnoy fixative series. These two fixatives bring about no change in the nucleic acid as far as can be

measured by the Feulgen reaction. The curve shows a rapid rise in stain intensity with increasing hydrolysis, until at 4 minutes a strong stain is reached. This maximum is maintained through the range from 4 to 8 minutes, then there is a slight dropping off in color to medium strong at 9 minutes and to medium at 10 minutes. The dropping off continues gradually until 15 minutes, then less rapidly to 20 minutes, and at 30 minutes' hydrolysis no stain is perceptible.

The second type of curve (fig. 1*B*) is shown by the La Cour 2 BE and Belling fixative series. This curve is very different from the other type. Instead of the color beginning to shade off at 9 minutes of hydrolysis it continues strong and heavy right through and past 30 minutes. Another difference is that the maximum is not reached so soon. At 5 minutes' hydrolysis the stain is medium strong; at 6 minutes it is strong, and this maximum strength is maintained until about 50 minutes when a falling off in intensity occurs.

This difference is apparently caused by some constituent or constituents present in the La Cour 2 BE and Belling fixatives but not present in the Feulgen and Carnoy fixatives. The only constituent common to both of the former fixatives but not present in the latter two is chromic acid. To make sure that chromic acid is the agent responsible, a series in which 1 per cent chromic acid was used as the fixative was treated in the same way. This produced the same type of staining curve as the La Cour 2 BE and Belling fixatives. Each of the other ingredients of the fixatives was tested individually and all produced the same type of curve as the normal or Feulgen and Carnoy types. There can therefore be no doubt that chromic acid and it alone is responsible for the marked difference in the staining curves.

DISCUSSION.—The *in vitro* experiments have given results confirmatory in a general way to those obtained by BAUER. There are differences, probably due mainly to the fact that it is easy to observe slight color differences in the agar blocks while in microscopical preparations their exact determination is difficult. In addition, the *in vitro* experiments permit an analysis of the action of the various ingredients of the fixatives. This BAUER did not do, and he failed to recognize that chromic acid is the cause of the two types of

staining curves. Thus his tentative explanation for the fixation effect cannot stand. To facilitate comparison and discussion, BAUER's curves are reproduced here as figure 2.

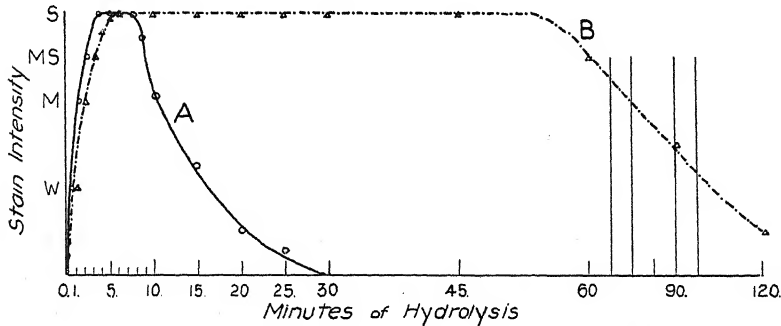


FIG. 1.—Staining curves as shown by nucleic acid impregnated agar blocks: A, Feulgen fixative (sublimite-acetic) and Carnoy (alcohol-acetic); B, La Cour 2 BE (chrom-osmic-acetic) and Belling (chrom-formol-acetic).

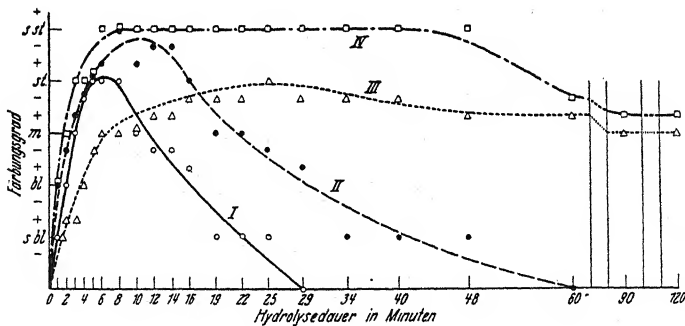


Abb. 1. Zusammenhang zwischen Hydrolysedauer und Färbungsgrad. Kurve I ○—○ Sublimatessigfixierung; Kurve II ●—● HELLY-Fixierung; Kurve III △—△ FLEMMING-HERTZ-Fixierung; Kurve IV □—□ SANFELICE-Fixierung.

FIG. 2.—Reproduction of BAUER's curves. Zeitschr. Zellf. Mikr. Anat. 15:231. 1932

In BAUER's curves, curve I corresponds to the Feulgen fixative curve and also to the Carnoy curve. Figure 1A represents the curves for both these fixatives. Curve II cannot be considered because the Helly fixative was not used in my experiments. Curve III corresponds to the curve for the La Cour 2 BE fixative and curve IV to the Belling fixative. These are both represented by figure 1B, since

with the agar blocks these two fixatives produce the same type of curve. The main difference in the two sets is that with the agar block experiments the maxima are all the same, whereas they vary in BAUER's experiments. This can probably be explained on the basis of the difficulty of distinguishing fine color differences in microscopical preparations. Why, in curve III, BAUER ignored the course of five points to go up to a maximum at 20 minutes is not understood. This curve, if it followed the majority of points, would show a flat topped maximum as in curve IV, just as is shown by the curves for a similar fixative in the agar block experiment.

BAUER's explanation that the rising phase of the curve is due to the gradual splitting off of the purine bodies by hydrolysis is chemically correct. For the falling off of the curve he gives two possibilities. First, it may be due to a further degradation of the thymine acid molecule (the part of the thymonucleic acid molecule left after the splitting off of the purine bodies); second, it may be due to the destruction of the union between nucleic acid and the protein components of the nucleoprotein. The second explanation is not tenable because the falling off of the stain occurs with the agar blocks just the same as it does with nuclei, and the nucleic acid is not bound to nucleoproteins in the agar blocks.

In the first explanation BAUER does not make clear what he means by a further degradation of the thymine acid molecule. It cannot be due to a chemical degradation of the aldehyde or color-producing groups, because a solution of thymus nucleic acid in a test tube is still capable of reaction and producing color after 8 hours of hydrolysis. To test this question further a series of nucleic acid blocks were hydrolyzed for 5-30 minutes in vials. The HCl from each vial was poured off and tested with leuco basic fuchsin at the same time that the corresponding agar blocks were tested. The acid in the vials showed a stain intensity which was the reciprocal of that of the agar block series. In other words, the aldehyde had passed out of the blocks into the hydrolyzing liquid. This experiment, together with the fact that 8 hours' hydrolysis does not bring about chemical degradation of the aldehyde groups, shows that a linkage is broken somewhere. Since the breakdown of the bonds is not between the nucleic acid molecules and nucleoproteins, and not within the alde-

hyde groups, it must be a breakage of the union between the aldehyde groups and the remainder of the thymonucleic acid molecule. Perhaps future biochemical investigation will reveal the details of this.

With the second type of curve the agar block experiments have shown that retention of the stain after long hydrolysis is due to chromic acid. Thus BAUER's explanation, which is based on the supposed unavailability and resulting unreactability of nucleic acid due to dense precipitation of the nuclear proteins brought about by the homogeneous fixation (gelatinization) of osmic and formaldehyde fixatives, as contrasted with the coarse coagulation of other fixatives, falls to the ground. BAUER, however, does not overlook the possibility of the effect of chromic compounds and the action of a specific chemical factor. The before-mentioned experiments to determine the reason for the fall in the curves would suggest that chromic acid acts in some way to strengthen the binding between the various groups of the nucleic acid molecule, allowing them to resist for a longer time the disintegrating effect of hydrolysis. This remains a subject for future investigation.

Besides the effect of chromic acid, the agar block experiments show, as BAUER has pointed out, that the Feulgen nucleal stain is not limited to material fixed in sublimate fixatives, which are in general unsatisfactory for cytological purposes. FEULGEN's precautions regarding the use of fixatives containing formalin or substances that are strong oxidizing agents like chromic acid are not necessary, provided these substances are in the concentration normally used in fixatives. The effects of the various other constituents of fixatives will now be discussed.

Osmic acid does not interfere with the stain if the darkening due to it is completely bleached out with hydrogen peroxide before hydrolysis. When it is not completely bleached out the stain is less intense.

Formalin, being itself an aldehyde and capable of reaction with leuco basic fuchsin to give the same color as the nucleal stain, was considered by FEULGEN to be an unsatisfactory constituent of fixatives as the color produced by it on the surface of tissues and in the cytoplasm would be apt to obscure the stain of the nucleus.

Such objectionable effects can be avoided by thorough washing following fixation with a fixative containing formalin. Proof that formalin can be thoroughly washed out is provided by the agar block experiment with 20 per cent formalin, in which the normal staining series is produced. Thus the reports of BERG (3), FEULGEN (8), HUBER (14), and VOSS (29) that formalin is an unsatisfactory constituent of fixatives seem untenable.

Some workers have claimed that fixatives with a low acetic acid content are best for use with the Feulgen reaction. SEMMENS and BHADURI (24), for their work on the differential staining of nucleoli and chromosomes, suggest Navashin's or Levitsky's fixatives with the acetic acid cut down to a minimum. Why, they do not say. WHITAKER (32), working with various plant materials, states that in his experience it is quite important that a fixative be employed which contains a minimum of acetic acid, and suggests Benda's (low acetic) fluid. In this laboratory we can see no difference in the intensity of the stain after using fixatives containing either a low or a high content of acetic acid. In fact some of our clearest and sharpest preparations were fixed in Carnoy's fluid (3:1), which contains 25 per cent of acetic acid. In order to test the effect of various concentrations of acetic acid on the nucleal stain, nucleic acid impregnated agar blocks were nucleal stained, after 24 hours' treatment, in 1, 5, 10, 25, 50, 75 per cent, and glacial acetic acid. All strengths up to and including the 50 per cent acetic acid gave the full maximum stain. With 75 per cent a slight reduction in the intensity of the stain was perceptible, and with glacial acetic acid a medium strong stain was produced. Thus all the evidence indicates that acetic acid has no deleterious effect on the Feulgen nucleal stain. As has been pointed out, the same statement applies to the other constituents of the commonly used cytological fixatives.

## II. EXPERIMENTS WITH PLANTS

In order to determine whether the nucleal stain is satisfactory for all types of plants and whether fixatives have the same effect as with the agar blocks, a similar series of experiments was carried out on various members of the different divisions of the plant kingdom. Particular attention was paid to those plants in which a nega-

tive reaction had been reported. Nuclei from as many different types of cells as could be obtained were studied in order to compare the stain in nuclei of different organs of the plant.

RESULTS.—The following plants were fixed in the four fixatives and hydrolyzed and stained in the same way as the agar blocks.

Cyanophyceae

*Oscillatoria*—stained granules

Diatomeae

*Navicula*, etc.—somatic nuclei

Chlorophyceae

*Ulothrix zonata*—somatic, zoospore, and gamete nuclei

*Cladophora*—somatic nuclei

*Spirogyra*, 4 species—nuclei

*Desmids*, *Closterium*, etc.—somatic nuclei

*Nitella batrachosperma*—somatic, egg, and sperm nuclei

Phaeophyceae

*Fucus spiralis*—somatic, egg, and sperm nuclei

Phycomycetes

*Mucor*—hyphal, immature, and mature spore nuclei

Ascomycetes

*Geopyxis cupularis*—hyphal, immature, and mature spore nuclei

Basidiomycetes

*Aleurodiscus canadensis* (Jackson, in ed.)—dividing and resting nuclei

Bryophyta

*Riccia natans*—spore mother cell and spore nuclei

*Marchantia polymorpha*—somatic, sperm, and egg nuclei

*Mnium*—somatic, sperm, and egg nuclei

Pteridophyta

*Pteris cretica crustata*—spermatozoid, prothallial, and somatic nuclei

*Adiantum cuneatum*—spermatozoid, prothallial, and somatic nuclei

Gymnosperms

*Pinus austriaca*—somatic, egg, and pollen mother cell nuclei

*P. sylvestris*—somatic, egg, and pollen mother cell nuclei

Angiosperms

*Lycopersicum esculentum*—somatic and pollen mother cell nuclei

*Typha latifolia*—somatic nuclei

*Tradescantia virginiana*—somatic and pollen mother cell nuclei

*T. paludosa*—somatic and pollen mother cell nuclei

*T. reflexa*—somatic and pollen mother cell nuclei

*Lilium harrisii*—somatic, pollen mother cell, and egg nuclei

*L. longiflorum* var. *formosum*—somatic, pollen mother cell, and egg nuclei

Also many other species of angiosperms.

DISCUSSION.—A positive nucleal reaction was obtained in all the plants listed. In the majority of cases the type of staining curve with a particular fixative corresponds with that for the similarly treated agar blocks. The only plant that did not conform more or less to the typical staining curves was *Spirogyra* spp.

In the case of *Spirogyra* the maximum is shifted to a longer period of hydrolysis (15–20 minutes). YAMAHA and SUEMATSU (33) give for this form a hydrolysis period of 5–10 minutes, and GEITLER (12) 5–8 minutes; in my experiments this range of hydrolysis did not produce a good stain. A negative reaction for *Spirogyra* has been reported by HUREL-PY (15), KIESEL and DOINIKOWA (16), MILOVIDOV (20), PETTER (22), and SHINKE and SHIGENAGA (26). GEITLER was the first to detect a positive stain. He investigated the structure of the resting nucleus and found that the stain was confined to widely separated small dotlike and rod-shaped bodies, the pro-chromosomes or chromocenters. It is possible that the negative results previously reported for *Spirogyra* are due to the fact that the investigators overlooked the small chromocenters, or perhaps they did not hydrolyze long enough to make them evident.

*Marchantia* was the only plant in which difficulty was experienced in obtaining a satisfactory stain with all types of nuclei. The staining curves were of the normal type but the maximum stain was weak. This weak stain occurred in all types of nuclei, even those of spermatozoids, which usually take the heaviest stain owing to the compactness of the chromatin. Results such as this would lead one to suspect a chemical difference in the nuclei of this plant.

It was observed in plants with which it was difficult to obtain an intense stain owing to the dispersed nature of the staining material that the best results were obtained after the La Cour and Belling fixatives. These fixatives have also the advantage that the period of hydrolysis is not limited to a narrow range as with the others. BAUER noted this and brought out another interesting point in favor of these fixatives. The Feulgen nucleal stain has frequently been criticized on the basis that the acid hydrolysis will perhaps destroy some of the finer structural details of the nucleus. BAUER points out that material fixed with fixatives which prolong the staining will maintain the true structure much longer because they resist

the effect of hydrolysis for a greater length of time. This applies to the chromic acid fixatives: In no instance in my work on the structure of chromosomes has any destruction of the finer details of structure been noted. Of course only short hydrolysis periods were used, and no doubt structure would be destroyed with longer hydrolysis, especially with the Carnoy fixative. The various experiments have shown that a nucleal stain is possible with all kinds of plants, and the intensity of this stain and thereby its usefulness is dependent on the interrelation of two factors, fixation and hydrolysis.

### III. PERIOD OF HYDROLYSIS

The time of hydrolysis is the cardinal factor in the Feulgen technique. WHITAKER (32) remarks that the time allowed for hydrolysis is one of the critical points of the technique, but then makes the statement: "In general, it is governed by the length of the period of fixation, i.e., if fixation lasts 30 minutes, hydrolysis should extend over the same period." Thirty minutes' hydrolysis will produce a good stain with the chromic acid fixatives such as Benda, but not with those lacking chromic acid, and if fixation extends over hours, hydrolysis could not be carried on for the same length of time. FEULGEN (8) gives 4 minutes as the optimum time of hydrolysis and this is correct for the sublimate-acetic fixative he was using. BAUER (1) gives the optimum time for twenty-four fixatives. The agar block experiments have demonstrated that the differences are due to chromic acid. From these experiments two generalizations can be drawn: (1) with fixatives containing chromic acid the optimum hydrolysis period for maximum stain is 5-30 minutes; (2) with fixatives lacking the acid it is 4-8 minutes. Exceptions to this general rule are known, especially in lower plants. For example, in the case of *Spirogyra*, with fixatives lacking chromic acid the optimum hydrolysis period is shifted up to 15-20 minutes. Thus in cases where no stain is produced in the normal hydrolysis range, a stain can often be obtained by increasing the time of hydrolysis. Also with large quantities of material which lower the temperature of the acid, and with large masses of tissue where it takes some time for the acid to penetrate to the interior and raise the temperature there, it is necessary to increase the time of hydrolysis. From the results

of the various experiments it can be concluded that with higher plants and with most of the lower ones a satisfactory nucleal stain can be brought about by 5 minutes' hydrolysis, no matter what fixative is used.

#### IV. POSSIBILITIES OF A NEGATIVE NUCLEAR REACTION

A negative nuclear reaction could result from the action of compounds which can be designated as stain inhibitors. By stain inhibitors are meant those compounds which have been reported as hindering or preventing the occurrence of a Feulgen reaction, thus

TABLE 1

PERCENTAGE CHROMIC ACID CONCENTRATION	FIXATION		
	10 MINUTES	1 HOUR	24 HOURS
1.....	S*	S	S
2.....	S	S	S
5.....	S	S	Smear gone
10.....	S	S	" "
25.....	Smear gone	Smear gone	" "
50.....	" "	" "	" "

\* S=strong stain.

resulting in a partial stain or none at all. The first of these compounds to be considered is chromic acid. YUASA (34), working with ferns, claims that above a certain limiting percentage of chromic acid (1 per cent for most species) a negative reaction results. Attempts to duplicate YUASA's work, using *Pteris* and *Adiantum* leaves, yielded results that do not agree with his. At concentrations of 25 per cent and above the leaves disintegrated, as did smears of *Tradescantia virginiana* pollen mother cells. Table 1 shows the results with smears, all with 5 minutes' hydrolysis.

The nucleic acid impregnated agar blocks present a clearer picture of what happens (table 2).

Obviously the partially or completely inhibiting action of chromic acid at high concentrations is due to its strong oxidizing power, which destroys the nuclear material. Yet to do this it requires a very high concentration acting for a considerable time. At concen-

trations such as are used in fixing reagents, chromic acid has no deleterious effect; on the contrary it postpones the falling off of the stain with increased hydrolysis.

Tannins are considered in many cases to be the cause of a negative nucleal reaction with plants. MILOVIDOV (20) pointed out that tannins are present in many plant cells, particularly epidermal cells, and that in these cells there is a weakening of the stain. On this basis he would explain the results of BOAS and BIECHELE (4), who reported that the nuclei of the epidermal cells of 138 out of the 224

TABLE 2

PERCENTAGE CHROMIC ACID CONCENTRATION	FIXATION		
	10 MINUTES	1 HOUR	24 HOURS
1.....	S*	S	S
2.....	S	S	S
5.....	S	S	No stain
10.....	S	S	" "
25.....	S	M	" "
50.....	S	W	" "

\* S=strong stain; M=medium stain; W=weak stain.

plants investigated showed a negative nucleal reaction. As TISCHLER (27) points out, perhaps these negative results could better be explained on the basis of faulty technique, failure to recognize small amounts of nucleic acid, slight chemical differences, and unsuitability of the materials and methods. MILOVIDOV seems convinced that tannins interfere with the nucleal reaction by hindering the penetration of reagents. He substantiates his claims by considerable experimentation. In cases where he can trace the presence of tannin to an individual cell he finds a weakening of the nucleal reaction. With the series of angiosperms which he investigated, two showed a negative nucleal reaction and others a weak reaction. These occurred in plants known to contain considerable quantities of tannins. In *Fucus vesiculosus* he reports that there is a weakened nucleal reaction in cells surrounded by tannins, but this could not be confirmed in my own work with *Fucus*. To test further the hindering

action of tannins, MILOVIDOV soaked slides with sections on them in 2 per cent tannic acid and found a weakening of the stain. Likewise he showed that preparations treated in this way failed to take up gentian violet and methyl green. In presenting this impressive series of experiments and facts to support his claims regarding the inhibiting action of tannins, MILOVIDOV concludes by stating that more experimental work is necessary to confirm his assumptions.

In order to investigate thoroughly the question of the effect of tannins on the nucleal reaction, extensive experiments with plant material and agar blocks were carried out. In all cases the material was soaked in 2 per cent tannic acid for 24 hours, rapidly rinsed in water to remove only the excess of tannic acid, then transferred directly to the HCl for hydrolysis. Other concentrations of tannic acid were tried but the effect was the same, so a 2 per cent solution was used as standard. The results of these experiments are shown in the graphs of figure 3. Each curve shows the results for nucleic acid impregnated agar blocks as well as pollen mother cell smears of *Tradescantia paludosa*, since both produced the same results.

Graph 1 represents the curve produced by the Feulgen and Carnoy fixatives. The tannic acid curve is very different from the normal type: the maximum is not reached until 8 minutes of hydrolysis, and instead of falling away there it continues through to 30 minutes of hydrolysis, where a slight dropping off begins. This is similar to the effect produced by chromic acid. Tannins certainly do not inhibit the stain, except perhaps slightly at first.

Graph 2 shows the curves with La Cour 2 BE fixative. Here the only difference in the two curves is that the maximum is not reached until 12 minutes of hydrolysis. Again there is a slight inhibiting effect at first.

Graph 3 is for the Belling fixative and here there is a definite weakening of the stain all through the range of hydrolysis. The maximum stain is never attained; a medium stain is reached at 10 minutes and this level is maintained up to 30 minutes and beyond.

Comparing these three graphs and the constituents of the four fixatives that produce them, formalin is the only constituent that is present in the Belling fixative and lacking in the others. Graph 4 shows the type of staining with 20 per cent formalin. The inhibition

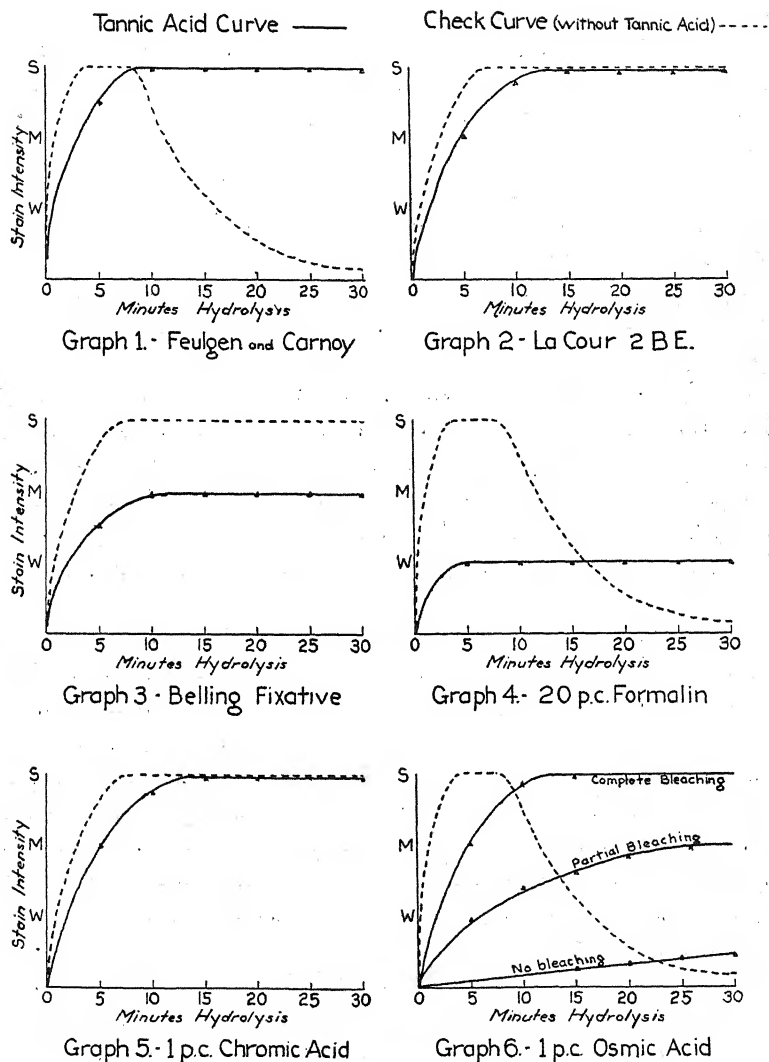


FIG. 3.—Effect of tannic acid on staining curves as shown by nucleic acid impregnated agar blocks and plant materials with various fixatives. Dotted line, check or normal result; solid line, tannic acid curve.

of the stain here is even greater than with the Belling fixative, as only a weak color is produced. This difference is probably due to the fact that 20 per cent formalin represents a higher concentration than that in the Belling fixative. That this general weakening is due to formalin alone can be seen by examining the various graphs. Graph 5 shows the type of curve produced by 1 per cent chromic acid and this is the regular chromic acid curve. The other ingredients of the fixatives—1 per cent potassium bichromate, 6 per cent sublimate, 5 per cent acetic acid, and 50 per cent alcohol—show the same type of curve as that for the Feulgen and Carnoy fixatives (graph 1).

Graph 6 is for 2 per cent osmic acid. The three tannic acid curves show the different amounts of inhibition produced by different amounts of bleaching of the osmic acid with hydrogen peroxide. The more complete the bleaching the sooner the maximum is reached. Lack of complete bleaching of the osmic acid probably explains the delay in reaching a maximum stain with La Cour 2 BE.

These results are in partial agreement with those of MILOVIDOV. In all cases there is a slight inhibition of the stain at the low periods of hydrolysis. These are the hydrolysis periods (4 to 8 minutes) normally used in cytological work, but the weakening is so slight that it may be ignored. The important point is that with fixatives containing formalin the weakening of the stain in its presence is serious. This is the sort of effect that MILOVIDOV reports, but he was not using fixatives which contain formalin. Thus the inhibiting action of tannin on the nucleal stain is not so serious as MILOVIDOV considers it to be.

Strong alkalies will inhibit the nucleal stain by virtue of the fact that they destroy the nuclear material. This was observed with root tips treated after hydrolysis with hot alkali to remove the pectic acid of the middle lamella in order to bring about a separation of the cells for the squash technique. This is discussed by HILLARY (13), together with the fading of the stain which results when corn syrup diluted with acetic acid is used as a mounting medium. This is the only instance as far as is known of the Feulgen nucleal stain fading.

The possibility of a negative nucleal reaction being reported owing to failure of the observer to see the widely separated chromocenters of resting nuclei has been discussed in the case of *Spirogyra*. This probably explains the report by WALTHER (30) of a negative nucleal reaction with the egg nuclei and a positive reaction with the sperm nuclei of *Nitella*. In my investigations the egg nucleus of *Nitella* showed small widely separated chromocenters. Another case of this sort was found in the nuclei of the germinating radicle of *Typha latifolia*, where the chromocenters were so small that the stain was hardly discernible; thus it would be easy to overlook them. The same was found to be the case with fungi; in most cases there was a large nucleus with small chromocenters distributed around the nucleolus and the periphery of the nucleus. A weak stain due to a great dispersion of the staining material in a large nucleus is strikingly demonstrated in *Fucus*. Here the ratio of volume of sperm to egg nucleus is 1 to 700, with presumably the same amount of staining material in each. The sperm nucleus shows a heavy stain while the egg nucleus shows a weak one because the stain is confined to the chromocenters, which are distributed over the large volume of the nucleus. A similar observation was made with the egg nucleus of *Pinus*, except that this nucleus shows a reticulate type of structure. A review of the literature dealing with this question in both plants and animals has been given by MILOVIDOV (20).

The final possible cause of a negative nucleal reaction is a chemical alteration in the thymonucleic acid. MILOVIDOV (20) has reviewed the literature dealing with this problem. In animals there is strong evidence to support the view that there is a chemical alteration or synthesis of nucleic acid during development. With plants the evidence is not so complete, but there are indications of it. WESTBROOK (31) finds a very weak reaction with the sporangia of *Rhodomenia palmata* during the growth stage. SHIMAMURA (25) reports a negative reaction with the reticulum of sperm and egg nuclei as well as with the central, ventral canal cell, and conjugating nuclei of *Cycas* and *Ginkgo*. This could perhaps be explained by the wide dispersion of the chromatin in the egg nuclei but not with the others, especially the sperm nuclei. BERG (3) claims a chemical difference to explain the negative reaction of the megagametophyte

nuclei in *Lilium*, but careful investigation of the megagametophyte nuclei at all stages of development has shown only a strong positive reaction, even in a well dispersed reticulum. A dispersion of the chromatin will not explain the very weak stain in all types of nuclei in *Marchantia*. The only explanation for this, excluding the possibility of stain inhibiting substances, is a chemical difference, and such could easily be the case considering the complexity of the nucleic acid molecule.

The possibility of the nucleal stain being obscured by colorations produced by leuco basic fuchsin in structures other than the nucleus was investigated. These colorations are produced (with or without hydrolysis) in lignin, suberin, cutin, starch, glycogen and other polysaccharides, owing to the aldehyde groups present in these compounds. (With no hydrolysis, this reaction is merely SCHIFF's test for an aldehyde.) The details of such stains have been discussed by BAUER (2), MARGOLENA (18), MIDDENDORF (19), and MILOVIDOV (20), so they will not be treated fully here. In only one case, *Vaucheria*, was the color in the cell wall strong enough to interfere with observation of the nucleal stain. In a few forms some trouble was caused by the plasmal reaction, which is a coloration of the cytoplasm. It is due to the action of leuco basic fuchsin on lipoids, which are retained by sublimate fixation (8) and chromic fixation (5). This can be overcome if the material is treated 24 hours with 95 per cent alcohol. These non-nucleal stains rarely interfere with the nucleal stain and need not be considered as drawbacks to the use of the nucleal reaction. On the other hand they provide additional uses for the stain; for example, the color produced by it on lignin is superior to that with phloroglucin.

The previous experiments and discussions show that there are various explanations possible for a negative nucleal reaction. These can be grouped under five headings, following MILOVIDOV's classification:

1. Unsuitable general methods; that is, insufficient fixation and preparation, or unsuitable object (presence of thick cell walls or heavy cuticle or fats which would hinder observation of the nucleus).
2. Incompleteness in the methods employed in the nucleal reac-

tion; that is, wrong period of hydrolysis, temperature of hydrolysis, and concentration of HCl.

3. Presence of materials which hinder the normal process of the reaction; that is, neutralization or binding of HCl; binding of the liberated aldehyde groups; disturbances of the penetration of reagents by resins, tannins, and slime substances (a slight effect).

4. Strong dispersion of a small amount of chromatin in a large nuclear volume.

5. Chemical alteration of thymonucleic acid.

The first four have been shown to be the reasons for negative results in many cases; the fifth, chemical alteration, while a reasonable possibility, still remains to be thoroughly investigated.

### Summary

1. In vitro experiments with nucleic acid impregnated agar blocks, using four different types of fixatives, show that the Feulgen nuclear stain gives two types of staining curves, depending on the presence or absence of chromic acid in the fixative.

2. With fixatives not containing chromic acid the maximum stain is produced by hydrolysis at 60° C. extending from 4 to 8 minutes; after that time a gradual falling off of the stain takes place, until at 30 minutes no further stain is visible. With fixatives containing chromic acid the maximum stain is produced by hydrolysis at 60° C. for 5-30 minutes.

3. That retention of the stain with increased hydrolysis is due to chromic acid has been demonstrated by testing the individual ingredients of the fixatives separately. In this case only chromic fixation produces the type of staining curve with the prolonged maximum.

4. None of the ingredients of the fixatives, if present at the concentration normally used, interfere with the stain. Chromic acid at high concentrations (above 5 per cent) and acting for a considerable length of time will prevent the stain, owing to destruction of the nuclear material.

5. Tannins, which have been considered by MILOVIDOV to inhibit the stain, have the same effect as chromic acid in retaining it, except

when a fixative is used that contains formalin. In that case there is considerable weakening of the stain.

6. Various plants from the different divisions of the plant kingdom, when treated with the four fixatives in the same way as the agar blocks, give the same results. A stain, usually a strong one, is obtained in all cases. In some cases the stain is not pronounced owing to dispersion of the chromatin in widely separated chromocenters or to the presence of a diffuse reticulum in a large nucleus. In one case, *Marchantia*, all types of nuclei show only a weak stain, suggesting a chemical difference, at least as regards part of the nucleic acid present. These are considered to be possible explanations of the frequently reported negative results.

7. It can be concluded in general that for the production of a satisfactory stain 5 minutes' hydrolysis with any of the regularly used cytological fixatives is sufficient.

The writer wishes to express his gratitude to Dr. L. C. COLEMAN, who suggested this problem and rendered generous assistance throughout its progress.

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# ONTOGENETIC AND ANATOMICAL STUDIES OF THE FLOWER AND FRUIT OF THE FAGACEAE AND JUGLANDACEAE<sup>1</sup>

LADEMA MARY LANGDON

(WITH SEVENTY FIGURES)

## Introduction

A critical study has been made of certain of the woody ament-bearing angiosperms, with particular attention to the Casuarinaceae, Fagaceae, and Juglandaceae. Life histories have been followed in detail, and all conclusions relating to floral structure have been fully supported by comparative studies of vascular organization. The principal object has been to determine what structures are primitive and what are reduced; that is, to secure additional data bearing upon the perplexing problem of the relationship of the various members of these families to one another and to the rest of the angiosperms.

An inconspicuous bractlike perianth, catkin development, and the frequency of chalazogamy placed the Casuarinales, Juglandales, and Fagales among the most primitive in ENGLER'S Archichlamydeae, preceding such petaliferous groups as the Rosales and Magnoliales. This position has been challenged by certain morphologists and systematists (3, 4, 9, 10, 12, 24, 35), who interpret the bractlike perianth and catkin development, both adaptations for wind pollination, as specialized rather than primitive features. The frequent occurrence of a syncarpous gynoecium among many of the Amnatiaceae is also considered by them to be at variance with the primitive position accorded these groups in the ENGLER system. Developed on the hypothesis that plants with a perianth associated with other primitive characters (floral and anatomical) are more ancient than plants lacking a perianth, HUTCHINSON'S new system

<sup>1</sup> A section of this report was presented before the General Section of the Botanical Society of America at Indianapolis; December, 1937. The investigation has been aided by grants from the Committee on Grants-in-Aid, National Research Council, and the Faculty Research Fund, Goucher College.

(24) derives the Amentiferae from the Magnoliales: the Juglandales along the line of the Celastrales and the Sapindales; the rest of the Amentaceae as reductions from the Rosales through the Hamamelidales; with the Casuarinaceae as illustrative of extreme reduction, possibly in adaptation to dry climatic conditions. In recent phyletic studies, BROWN (11) likewise inclines to the derivation of the Juglandales from the Rosalian line by way of the Sapindales, and interprets the cup-shaped torus characterizing the Rosales and many of their descendants as a nectary located between the carpels and the stamens. In the case of the Juglandales, he assumes a degeneration of the nectary along with the development of adaptations for wind pollination, a tendency to be observed also in the Aceraceae.

On the other hand, RENDLE's taxonomic studies (34) support the theory—as the one most in accord with present knowledge—that the Casuarinales, Fagales, and Juglandales are surviving representatives of one or more diverging lines of development from some early angiospermous type or types now extinct, very likely with bisexual hypogynous flowers and bractlike perianths. Summarized, his conclusions are that the complete absence of the herbaceous habit, the frequency of the chalazogamic method of fertilization, the marked interval between pollination and fertilization, as well as the long geological history of these orders suggest that in these forms we are concerned with isolated remnants of relatively ancient groups which have left no descendants among the more highly developed orders of our present flora. A similar conclusion appears to have been reached by NAVASHIN and FINN (30) with respect to the Juglandaceae.

Recent researches in inflorescence anatomy and morphology in families of the Amentiferae, including the Salicaceae (20), Betulaceae (1, 2), and Juglandaceae (28), have emphasized specialization and reduction in these groups; and those who have gone into the details of floral structure have been in general agreement in characterizing the inferior ovary as "appendicular." Opposed to this interpretation of the epigynous condition in flowering plants in general are the developmental studies of THOMPSON (40, 41, 42), those in paleobotany by THOMAS (38, 39), as well as the floral studies of others (13, 22, 23, 37) which give substantial support to the "re-

ceptacular" origin of the inferior ovary. Critical discussions of the theories of THOMAS and THOMPSON have appeared (5, 38, 44), and reference will be made to them at later points in this paper.

### Investigation

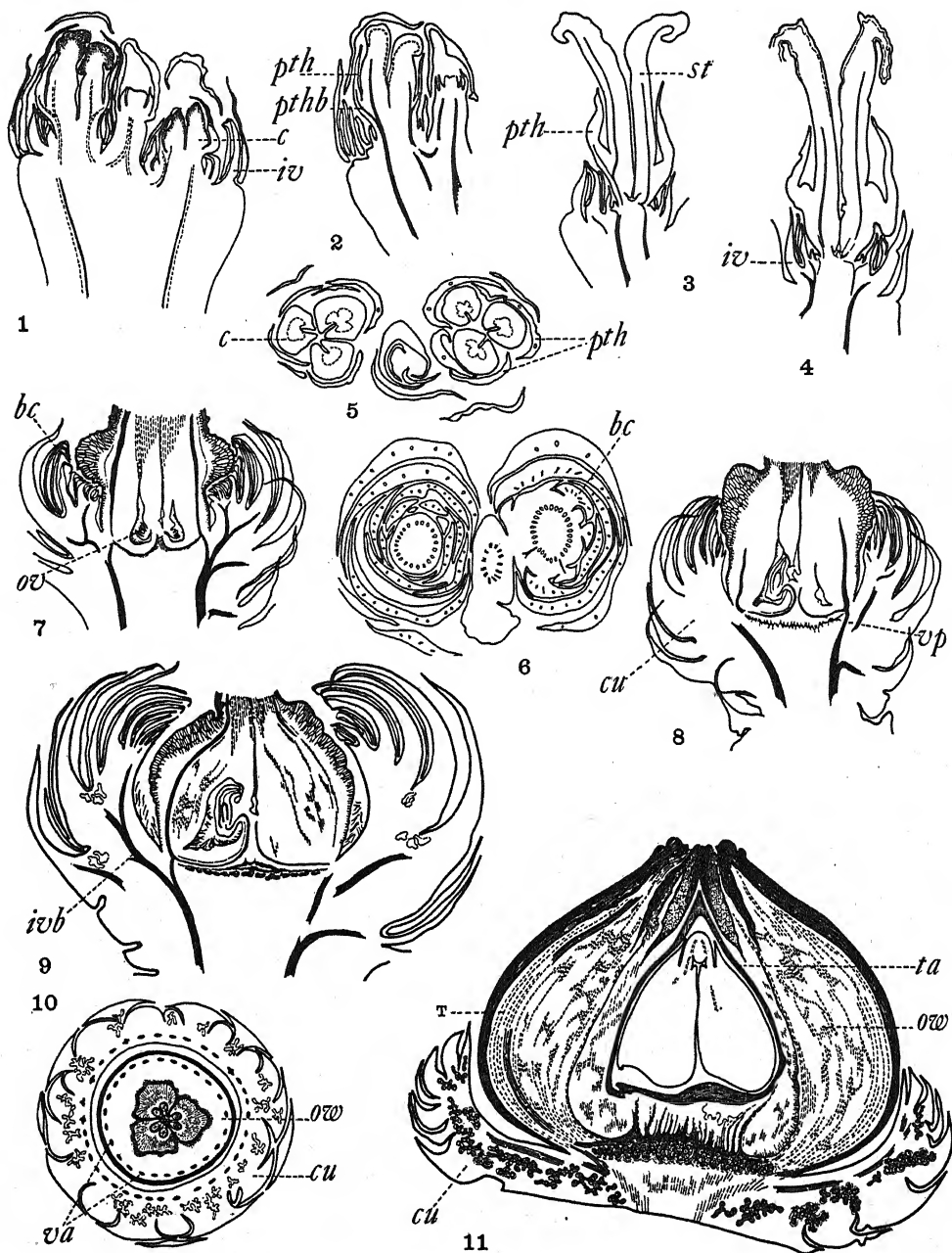
Two representatives each of the Fagaceae and the Juglandaceae—*Quercus rubra*, *Fagus americana*, *Carya glabra*, and *Juglans mandchurica*—have been selected for comparative treatments, and the conclusions are based upon an examination of serial sections of pistillate flowers and fruits at different developmental stages. Floral and embryological features of the Casuarinaceae will be dealt with in a later paper.

In the Fagaceae and Juglandaceae, collections of material for imbedding were taken at frequent intervals, from April 1 to early August, from trees on the campus and in the arboretum at Johns Hopkins University, and from the gardens of Dr. E. A. Andrews at Govans, Maryland. In all cases samples were secured from swelling buds and tips of growing shoots both before and following appearance of the pistillate flowers. Upon emergence of the young flower clusters, collections were made three to four times weekly to the time of pollination; after pollination daily for a week or two; and then at intervals of two to three days through June, July, and early August (periods of embryo and fruit development).

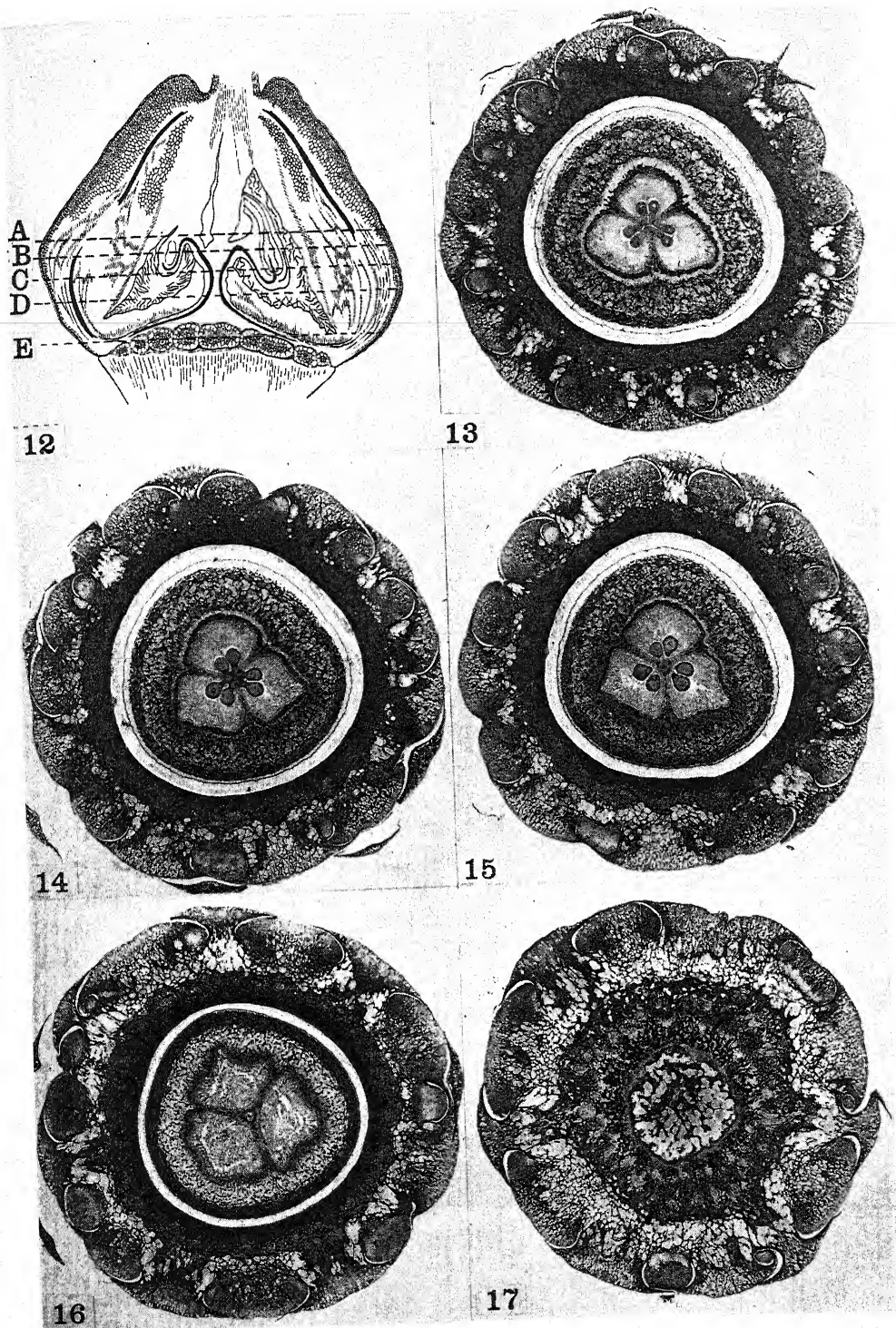
More than 500 fixations of inflorescences, flowers, and fruits were prepared and imbedded through three collecting seasons, and substantially the same technical procedure was followed as was reported in an earlier paper in this series (27). In preparing the fruits for paraffin imbedding, it was found advisable, after exhausting as much air as possible from the tissues, to substitute in dehydration mixtures of ethyl and n-butyl alcohols for the usual ethyl alcohol-xylene series (45). Serial sections 10–12  $\mu$  in thickness were secured with a Spencer sliding microtome (26). Only by this method has it been possible to procure the preparations required for these studies.

### I. FAGACEAE

While the morphology and anatomy of the inflorescences and flowers of the Betulaceae have been studied more or less intensively



FIGS. 1-11.—*Quercus rubra*. Figs. 1-6, longi- and transections of young pistillate inflorescence and individual flowers, early to late May (1st season). Figs. 7, 8, young fruits, early to late May (2d season). Fig. 9, solitary fruit enveloped by bracteolate involucre. Fig. 10, transection of young fruit within cupule; distribution of vascular tissue both in cupular and floral axes shown. Fig. 11, partially matured fruit within cupule; testa of solitary seed incloses well developed embryo (bc, involucre scales; c, carpels; cu, cupule; ivb, involucre bundles; iv, involucre; ov, ovule; pl, placental axis; pth, perianth; pthb, perianth bundles; st, style; ta, testa; fva, vascular axis of ovary; vp, vascular supply to placenta; ow, ovary wall—axis+non-diverged pericarp).



FIGS. 12-17.—*Q. rubra*. Fig. 12, longisection of young fruit freed from enveloping cupule. Figs. 13-17, structure of fruit, including cupule, in transections at levels A, B, C, D, E of fig. 12.

by several investigators, including the recent detailed treatment of their inflorescence anatomy and morphology by ABBE (1, 2), similar studies involving the Fagaceae have been, since the early taxonomic and phylogenetic treatises (18, 33, 36), conspicuously meager. RENDLE (34) gives a more complete comparative account of generic differences in the family than are found in other recent phylogenetic studies of the flowering plants, but there is a noticeable deficiency in detail as well as scarcity of illustrations, with most of the latter taken from earlier works. BENSON's contributions (7) were concerned chiefly with the megagametophyte in genera of the Betulaceae and Fagaceae; they added little to their floral morphology. Summarizing a comparative account of the archesporium, BENSON concluded that the Cupuliferae were less specialized than the Betulaceae, thus supporting EICHLER's deductions based on floral morphology.

The Fagaceae generally are monoecious. The male flowers, in slender catkins or small capitate clusters, are borne usually in upper scale-leaf axils of the shoots of the current year; the female flowers, as solitary florets or 2- or 3-flowered cymose inflorescences within scaly, cuplike involucre, arise in the upper foliage leaf axils. Exceptions to this arrangement are found in the androgynous aments of *Pasania* and *Castanea*. From the transition region of the upper androgynous aments of the latter, a variety of inflorescence types have been reported (18), from 7-flowered staminate cymes with staminodia and pistil rudiment only in the primary florets to 3-flowered pistillate cymes all flowers of which are bisporangiate. In *Fagus*, staminate florets have been observed occasionally in the axils of upper leaves of the involucre of pistillate inflorescences, with the two normally oriented flowers of the inflorescence both bisporangiate (figs. 20, 32). Mixed inflorescences likewise are reported in genera of the Betulaceae (2), as well as the exceptional case of the replacement of individual flowers by miniature aments within the pistillate catkins of *Alnus*. Both in *Quercus* and *Fagus* the staminate florets not infrequently bear pistil rudiments.

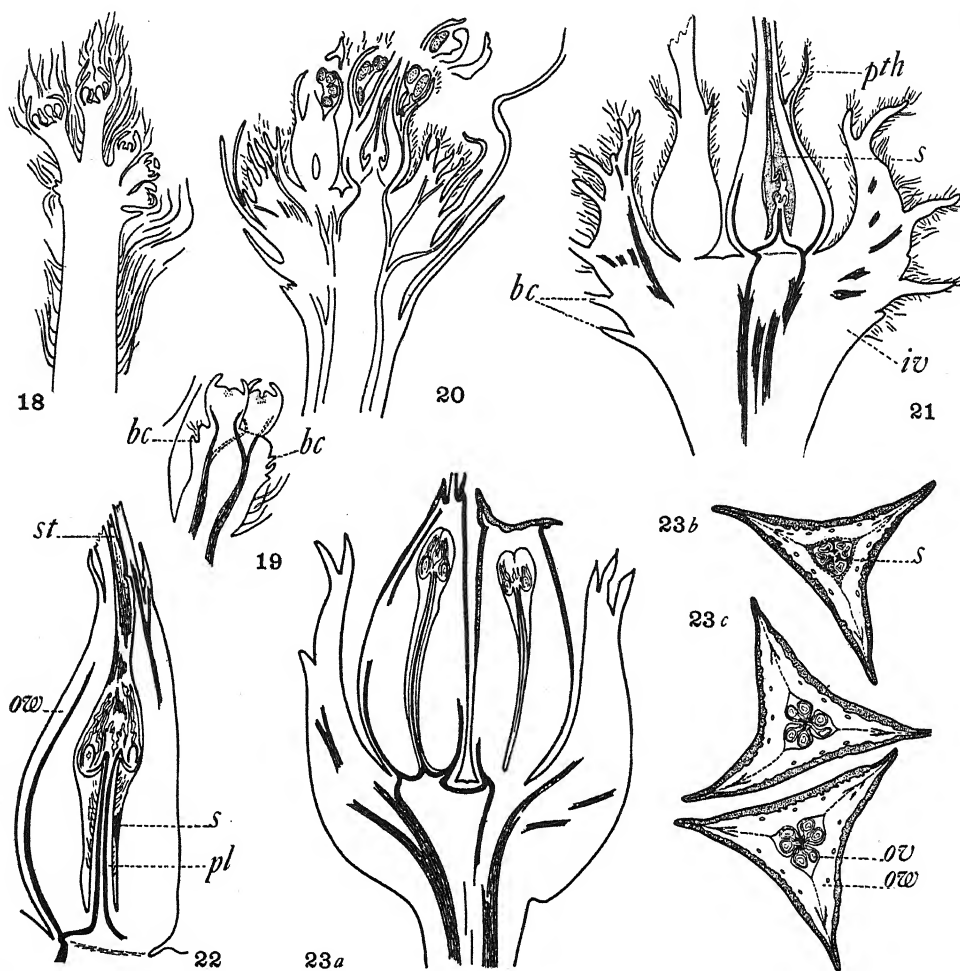
Familiar differences in the flowers and fruits of the six genera of this family arise from the various developments of the involucre and the number of flowers or fruits which it surrounds; for example, in

*Castanea* and *Fagus* four scale-bearing involucre segments inclose a 2- or 3-flowered inflorescence, while in the other four genera the female flowers (solitary or as 3-flowered cymes) are inclosed by a cupular scale-bearing involucre.

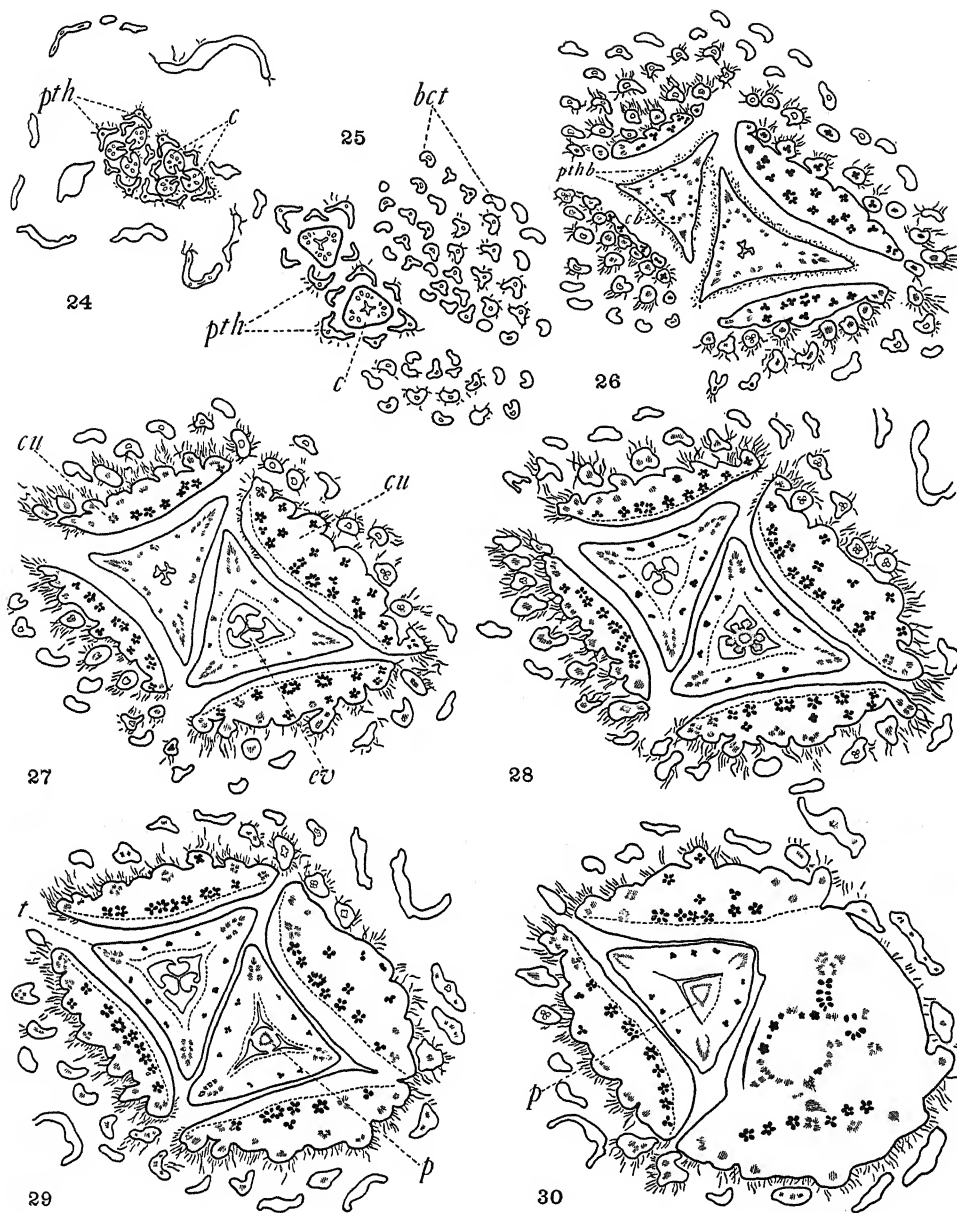
FLOWER AND FRUIT DEVELOPMENT IN THE BEECH.—Taxonomic accounts generally agree in describing the individual female flower as consisting of a 6-lobed calyx adnate to the 3-celled ovary, with as many styles as its cells, and one or two pendulous semi-anatropous ovules in each cell. These flowers, usually two together, are inclosed more or less completely by a 4-lobed, bracteolate involucre, the four basal bracts of which are longer than the involucre segments. Differences of opinion arise chiefly with regard to interpretation of the ovary and its placentation, and also as to the character of the involucre.

In the ontogeny of the pistillate inflorescence of *Fagus americana* the two florets are differentiated at the apex of the peduncle well in advance of development of the swollen bract-bearing portion of the axis beneath the flowers. In the young inflorescence illustrated in figure 19 both flowers already bear the primordia of perianth leaves and carpels, the latter appearing to arise in a slight depression of the receptacle and alternating with the inner set of perianth leaves. Rapid growth of the tissue underlying the three outer sets of floral members results in a fleshy, cupular perianth tube lined by the non-diverged carpellary tissue (figs. 20, 31, 33).

In the meantime, the portion of the floral axis inclosed by the carpels produces a short, thick columnal structure, at the tip of which ovule development is initiated. Centripetal growth of the incurved carpel margins takes place concurrently with development of the placental axis, and as cushion-like masses of parenchyma they envelop the placentae and ovules (fig. 21). Above the ovules and placental axis the carpellary ridges expand as parenchymatous wedges (figs. 27 and 29, *ev*) which meet in the center of the ovarian cavity but do not unite. Directly beneath the ovules the carpellary ridges grow conjointly with the placental axis (figs. 28, 29), thus forming the septa of the trilocular ovary. It is to be noted at this juncture that although carpellary tissue supports the placental column and closely invests the developing ovules, it does not bear the



FIGS. 18-23.—*Fagus americana*. Fig. 18, young staminate inflorescence showing median and one of lateral groups of florets. Fig. 19, young pistillate inflorescence. Fig. 20, pistillate inflorescence about April 15, consisting here of two bisporangiate and single staminate floret, latter appearing only in part. Fig. 21, young fruits inclosed by involucre segments. Fig. 22, single fruit showing vascular supply to placental axis in its relation to floral vascular axis, also to ovules. Fig. 23a, young fruits with investing involucre segments about June 15. Fig. 23b, c, transverse sections of upper portion of two fruits illustrated in fig. 23a, b, above point of attachment of ovules, and c, at point of insertion of ovules on placental axis (bc, involucre bracts; iv, involucre; pl, placental axis; pth, perianth; ov, ovule; ovw, ovary wall; s, sepal tissue; st, styles).



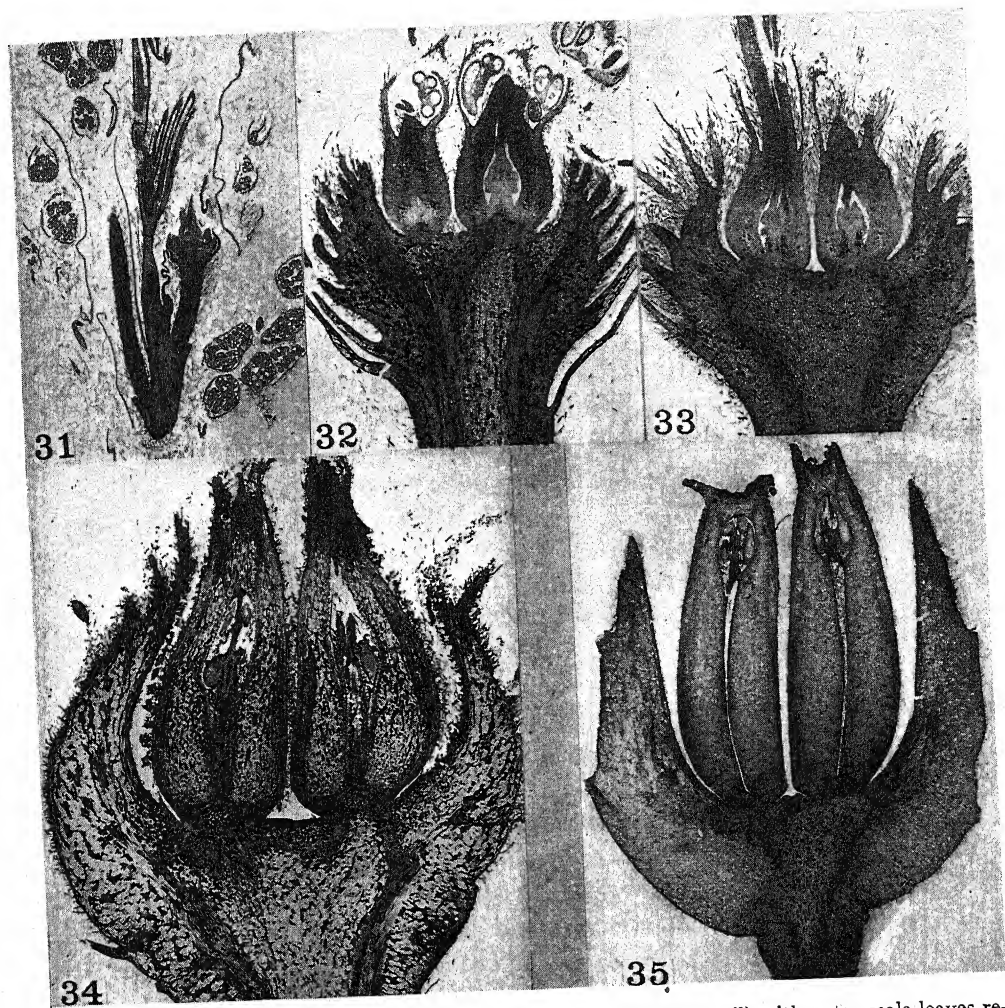
FIGS. 24-30.—*F. americana*. Transsections of pistillate inflorescence at different levels from tip to base showing arrangement of vascular traces of florets and involucre and their connection with vascular axis of peduncle (*c*, carpels; *cb*, carpellary bundles; *cu*, cupule segments inclosing the two triangular florets; *bc*, involucral scales; *ev*, centripetally growing carpellary tissue; *ov*, ovules; *pth*, perianth; *pthb*, perianth bundles; *p*, placental axis). Dotted lines within florets differentiate axial tissue from inner carpellate portion of ovary wall. Shaded structures in center of right floret of fig. 28 are differentiating ovules.

ovules nor is it supplied with vascular tissue. Furthermore, with the inception of fruit development the carpellary ridges are withdrawn from the vicinity of ovules and placentae by expansion of the ovary wall (fig. 23*a*, *b*), creating an incompletely trilobulate condition in the upper part of the ovary.

The formative phases outlined in the preceding paragraph, excepting those of the last statement, take place before the opening of buds and emergence of the pistillate heads early in April. BENSON (7) likewise observed that in young female flowers of *Fagus sylvatica* examined early in April "ovules are already laid down, and the rudiments of the inner integuments seen to be forming." At anthesis (April 15-20) the state of ovary and placental development illustrated in figures 21 and 33 is reached. Subsequent development of the ovary wall and shaftlike placental axis just about keep pace, with a gradual shifting of meristematic activity toward the basal section of both; and by the mid-part of May the flowers present the structural features illustrated in figures 22 and 34. The ovules, now in an advanced state of development, have curved to an erect position, with micropyle and chalaza at right angles to the funiculus.

The manner of distribution of the vascular tissue to the ovary wall and placental axis is shown in figures 20-23, and as transections in figures 24-30. Each pistillate flower is supplied directly from the vascular axis of the peduncle with a vascular cylinder consisting of eighteen compound bundles, some distributed along the sides of the triangular floral axis while others are closely associated at the ridged angles of this axis. From each of these vascular units, strands to the axile placentae diverge at the base of the ovary. Within the placental column the placental bundles form a triangular vascular axis extending to the point of insertion of the ovules. In no case have they been observed to extend beyond that point. The perianth and styler traces, as conjoint bundles, proceed from the units of the floral vascular axis through the marginal part of the ovary wall to the point of their divergence either to perianth or styler members.

By the latter part of June or early July the young fruits of the beech, inclosed by spiny, coriaceous involucreal segments, present the structural features illustrated in figures 23*a* and 35. Vestiges of



FIGS. 31-35.—*F. americana*. Fig. 31, section of young bud (early April) with outer scale leaves removed to reveal flower clusters, here consisting of one pistillate and portions of two staminate aments. Fig. 32, same type of inflorescence as in fig. 20. Fig. 33, typical 2-flowered pistillate inflorescence at pollination. Figs. 34, 35, young fruits invested by involucre.

perianth and styler members appear as partially lignified appendages at the tips of the angular fruits. Lignification is evident also at the periphery of the fruit (nut), as also is the tomentose coating of the inner surface. The ovules suspended from the tip of the hairy placental axis with micropyles directed toward the apex of the ovarian cavity are found to be in various stages of abortion, only one, occasionally two, bearing normally enlarged megagametophytes.

Characterization of the pistillate flower of the Fagaceae, based on evidence furnished by ontogeny and anatomy, as an ovule-bearing axis closely invested by a cupular envelop, the latter the product of conjoint growth of stem and carpels, calls to mind conclusions reached by THOMAS (38) following a survey of the reproductive structures of the megaphyllous seed plants. He concluded that the flower commenced as a sorus or tuft of sporangia terminating a branch, this seed-bearing branch becoming more and more completely inclosed by a carpellate structure formed from the conrescence of cupules. The idea of the origin of seeds from a sorus-like structure in which only the central sporangium remains fertile has received support from paleobotanical researches, and is made the basis of an illustrated series by CHAMBERLAIN (14), who has depicted the hypothetical development of the seed condition in the Cycadofilicales from an assumed heterosporous fern ancestry. CHAMBERLAIN's diagrams start with a sorus terminating a leaf, and progress through a series in the course of which the cupular indusium as well as the sporangium and megaspore walls become united to form the thick coat of the pteridosperm seed.

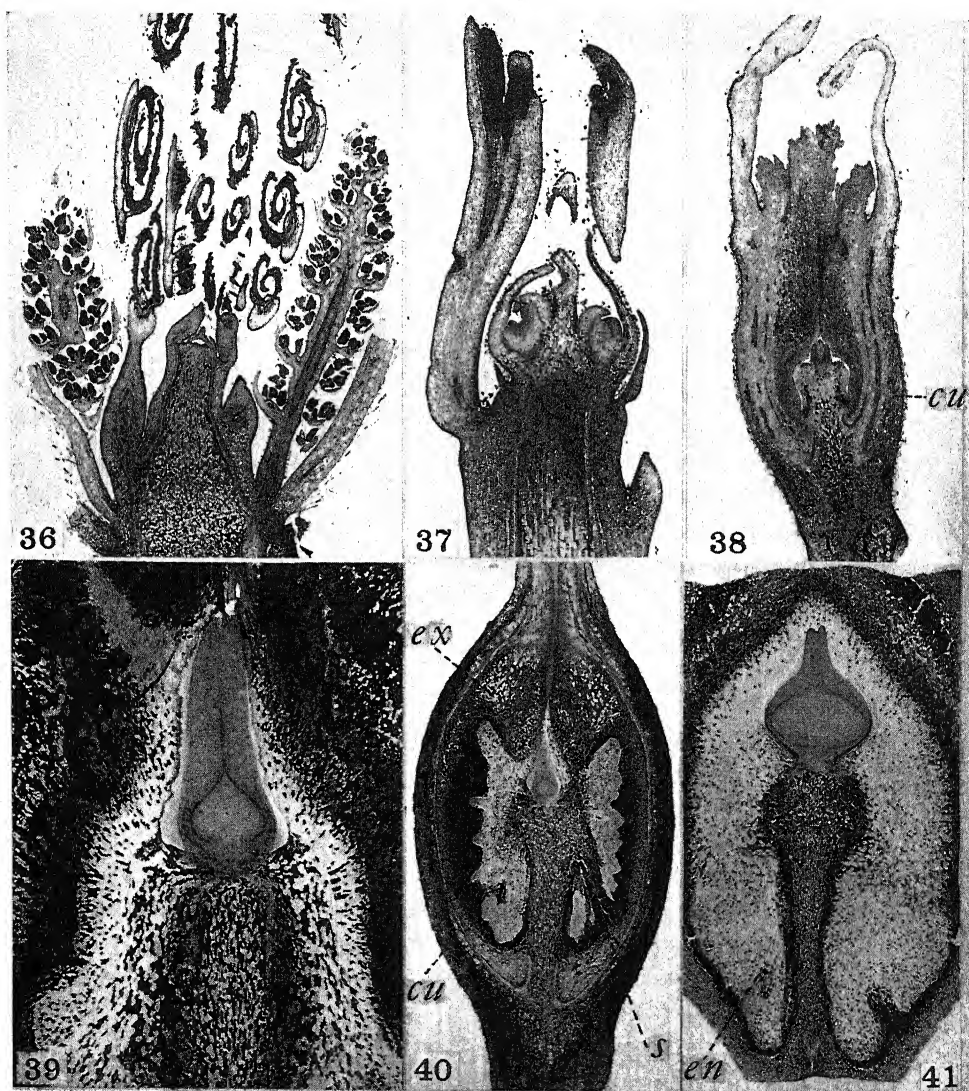
In supporting the theory of the evolution of the floral condition from a terminal sorus rather than from a condensed strobiloid body, THOMAS places special emphasis upon the aggregation of the sporangial structures at the tips of special branch systems.

PISTILLATE FLOWER AND FRUIT OF THE OAK.—The female flowers of *Quercus rubra*, each subtended by a bract and two bractlets, appear in short 2- or 3-flowered spikes from the upper axils of the leaves of the year. The individual flower, partially inclosed by a cupular involucre of imbricated scales, consists of a cup-shaped perianth tube non-diverged from the walls of an incompletely

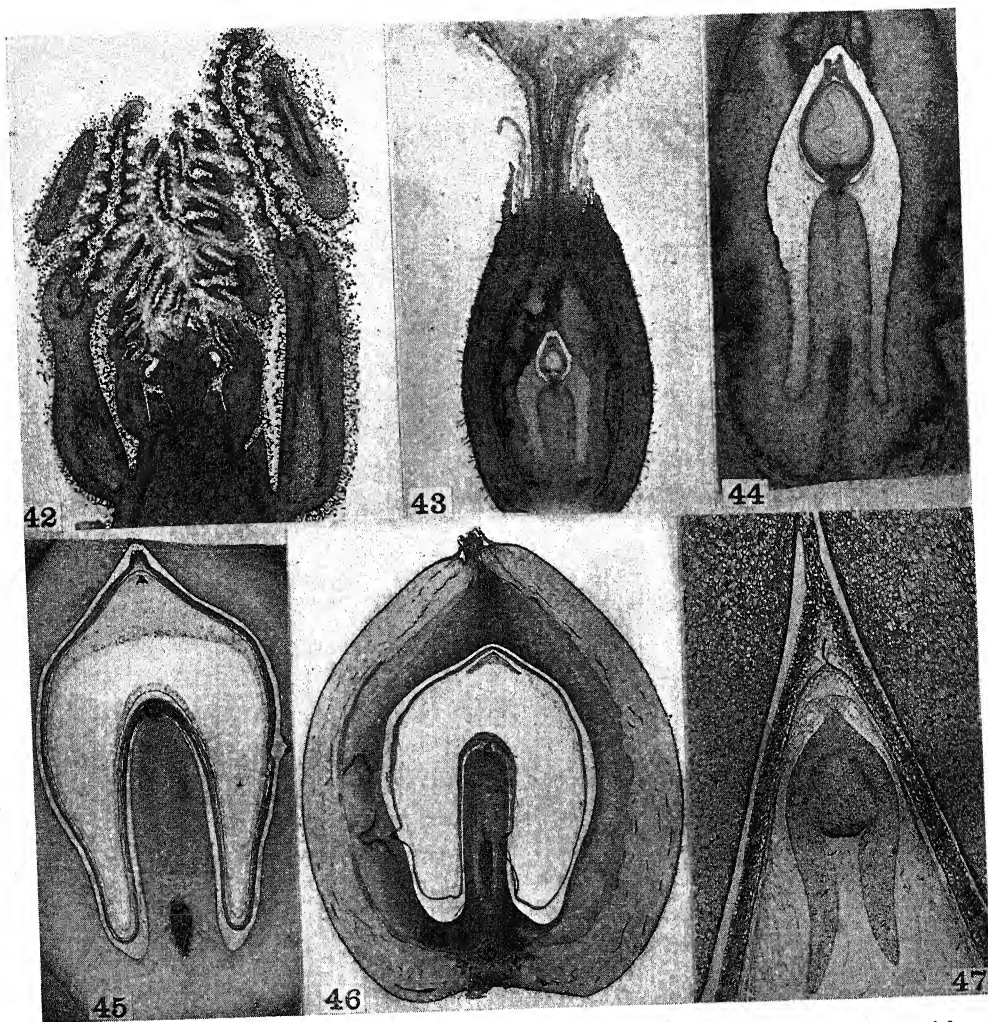
3-celled ovary, the latter bearing three erect styles stigmatic at their tips and along their inner surfaces. Development of the flower follows substantially the same sequence as that described in the beech. The character of the placentation and its origin are as observed in *Fagus*, but are somewhat more difficult to ascertain because of the more compact character of the placental axis and the prominent development of the centripetally growing ridges of carpellary tissue (figs. 4, 7, 8).

Anthesis and pollination in *Q. rubra* generally occur between May 7 and May 10 (figs. 1, 2, 3), but development of the placental tissue and formation of the ovules are not well advanced until the middle of June (fig. 4). At that time a strong development of procambium is evident in the vicinity of the placental axis, the strands differentiating along the base of the ovary to the placental column apparently as extensions of the floral vascular axis into the apical part of the flower. A single vascular ring of 20-24 bundles from which the vascular elements of both the perianth and the styler members are diverged, as also those of the placental axis, proceeds from the base of the ovary through the axial portion of the ovary wall (figs. 6-10, 13-15). The branching of the perianth traces from this ring occurs slightly below the level from which the styler traces are diverged.

From the latter part of July of the first season to early April of the second, only slight progress is made in the development either of ovules or of ovary. Only the bract-bearing involucre axis undergoes further development. As a fleshy cup-shaped structure, bearing at successively higher levels numerous scalelike leaves, it so completely incloses the flower through the fall and winter period that only the lignified vestiges of styles and perianth are distinguishable above its rim. By the early part of May of the second year (fig. 7), considerable expansion of the ovary wall is evident, as well as a deepening of the placental axis, and the ovules bear the rudiments of inner and outer integuments. A unique feature of the ovules, both of *Quercus* and *Fagus* but particularly marked in *Q. rubra* (and reported in the ovules of the magnolia), is the appearance in the elongated nucellus of a central several-layered strand of slender, procambium-like elements which extend through the base



FIGS. 36-41.—Pistillate flower and fruit of *Carya glabra*. Fig. 36, longisection of apical part of flower bud showing young staminate catkins; ovulate head as yet undifferentiate at apex of shoot. Fig. 37, young pistillate inflorescence; bracts of peduncle present. Fig. 38, median section of flower cut in plane of primary septum; ovule at archesporial stage. Fig. 39, ovule with mature megagametophyte. Fig. 40, median longisection of young fruit transverse to plane of primary septum, showing parenchymatous endocarp surrounding ovule and septum, also early differentiation of second septum which proceeds from two points—from margins of original septum and inner edge of carpel walls. Fig. 41, more advanced stage showing enlarged ovule at tip of shaftlike septum (*cu*, cupule; *en*, endocarp; *ex*, exocarp; *s*, secondary septum).



FIGS. 42-47.—*Juglans mandchurica*. Fig. 42, longisection of young pistillate inflorescence with enveloping foliage leaves. Fig. 43, median longisection of young fruit perpendicular to plane of septum, 5 days after pollination; pericarp clearly defined, also vascular tissue extending between involucre axis and ovary. Fig. 44, longisection of young fruit perpendicular to septum about 2 weeks after fertilization. Ovule shows megagametophyte with parietal layer of endosperm, also 18-20-celled embryo. Fig. 45, median section of nut perpendicular to septum 5 weeks after pollination; seed coat expanded to about full size, extending to all available space within the shell. Well defined layer of endosperm, cellular throughout, lines the seed coat, and embryo with cotyledons is distinguishable at apex of sac. Fig. 46, median longisection of nut closely invested by involucre axis (husk), sectioned perpendicular to primary septum. Fig. 47, enlargement of apical part of megagametophyte showing embryo as it appears sectioned in plane of carpels and primary septum.

of the nucellus and are continuous with the vascular tissues of raphe and funiculus. During the period of invasion and disorganization of the nucellar tissues by the megagametophyte, this procambial shaft is for some time a persistent and characteristic feature of the ovule. This and other features of the ovule and megagametophyte morphology of *Quercus* will be treated in greater detail in a later comparative embryological study of the Fagaceae.

The young fruit and one of the ovules as they appear at the time of fertilization, about June 16, are illustrated in figure 12. Development of the single seed and expansion and differentiation of the fruit coat are rapid from this stage, and by early July the fruit is half matured (fig. 11). In the developing wall of the fruit there is little if any distinction between the tissues of the pericarp and those of the conjoint stem (figs. 9-16). There is some lignification of the upper section of the pericarp, but a far greater amount of hardening takes place in the outer border of the axial section of the wall. The pericarp becomes desiccated and partially disorganized as seed development progresses (fig. 11).

Thus it appears that the fruits of the beech and oak meet the generally accepted definition of the "nut" type of fruit; that is, a hard, one-seeded, indehiscent fruit, usually produced from a compound ovary. Objection might be raised to this classification, however, on the grounds that these fruits develop from epigynous flowers, in which the hard outer rind is formed from non-diverged stem axis rather than from pericarp.

## II. JUGLANDACEAE

RENDLE (34) has called attention to the resemblances between the Fagales and the Juglandales—in the catkin arrangement of the unisexual flowers; in the similarity in general plan of flower structure, including the presence in some genera of a pistil rudiment in the male flowers; and in the major role played by bracts and bracteoles in the protection and distribution of the fruit. RENDLE believes that the epigynous perianth in the Fagales may be compared with the various stages of union of the perianth to the ovary in the Juglandales.

The pistillate catkins of the two species of the Juglandaceae

treated in this paper are solitary, terminal, and generally few-flowered; in *Juglans mandchurica* there are eight to ten in a spikelike inflorescence, while in *Carya glabra* there are but two or three (figs. 37, 42). Contrasting with the situation in *C. glabra*, where only two or three of the basal or first formed flowers of each inflorescence continue in development, is the situation in *J. mandchurica* where practically all the flowers in each ovulate head reach full development and are receptive at pollination. Furthermore, a large percentage of the flowers are pollinated and continue in fruit development (27). A comparative study of the inflorescence characters of the six genera and many of the species of the Juglandaceae has recently been made by MANNING (28), and an evolutionary series from primitive to advanced genera proposed on the basis of inflorescence types.

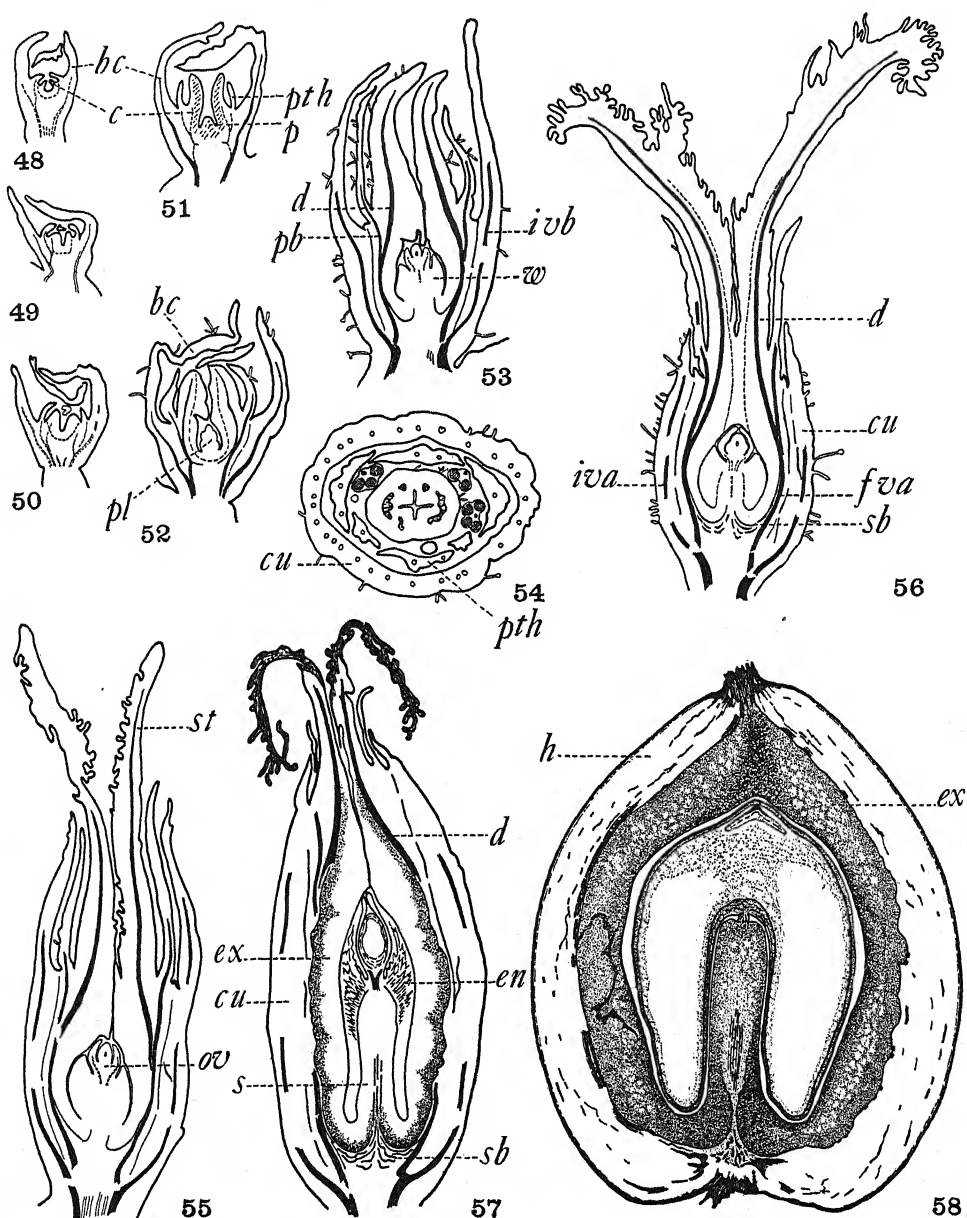
STRUCTURAL AND DEVELOPMENTAL FEATURES OF THE PISTILLATE FLOWERS OF JUGLANS AND CARYA COMPARED.—The female flower of *Juglans* and *Carya*, composed of a fleshy, 2-celled ovary (partly carpellate, partly axial in nature) crowned by an inconspicuous 1-4-leaved perianth and a pair of stigmatic styles, is inclosed by and united with a bracteolate, cupular involucre. Pistillate flowers of *Juglans* are generally described as bracted and 2-bracteolate with a 3-5-lobed (normally 4-lobed) calyx; those of *Carya* as bract fugacious, and calyx 4-lobed. According to other taxonomic statements, the female flower of *Carya* possesses a "perianth-like" involucre consisting of a bract and two bractlets in addition to one or two minute perianth leaves. In *Juglans* the involucre is united with the ovary to a little above the middle, while in *Carya* the involucre axis is united with the ovary to its apex.

The morphological character of the single orthotropous ovule of the Juglandaceae and its relation to the carpels have been discussed by several investigators, including DE CANDOLLE (15), VAN TIEGHEM (43), NAVASHIN (29), KARSTEN (25), NICOLOFF (31), BENSON and WELSFORD (8), and more recently by SHUHART (37) in a study of *Hicoria*. BENSON and WELSFORD's conclusions supported those of VAN TIEGHEM that the vascular supply for each of the floral leaves is given off from the stem-stele at the base of the flower, and that the ovary is therefore superior; also that the ovule though appar-

ently developed from the top of the floral axis is really an outgrowth from the carpels, one or both of which contribute to its vascular supply. With the exception of VAN TIEGHEM and BENSON, the investigators mentioned have arrived at the conclusion that the ovule in the *Juglans* group is a development from the top of a floral axis in the formation of which the carpels have no part. Ontogenetic and vascular studies combined in the present investigation support this conclusion.

In the developmental account of the pistillate flowers of the Juglandaceae, *Juglans mandchurica* will be described in some detail, with briefer reference to comparable stages or features in the ovulate flowers of *Carya glabra*. Figures 42-47 and 48-58 show median vertical sections of the pistillate flowers and fruits of *J. mandchurica*, the collections dating from the early part of April to early June. Sections of young pistillate inflorescences collected early in April show flower primordia in various stages of differentiation (fig. 42). These primordia arise acropetally on a short peduncle, terminating the season's growth, until each inflorescence is composed of eight to ten buds. Figure 42 also illustrates a striking and characteristic feature of the young flower buds of *J. mandchurica*—the remarkable exhibition of hairs, glandular intermixed with long, stiff protective ones, which cover both surfaces of the unexpanded leaves, peduncle axis, and the flower primordia. The female inflorescence becomes visible above the leaves of the shoot early in May.

Three protuberances, one a little in advance of the other two, are the first structures to arise from the outer circumference of the broad tip of the flower primordium. These are the primordia of the involucre leaves, consisting of a single bract and two bracteoles. They reach prominent proportions with some elevation of the immediately underlying tissue before appearance of the four perianth lobes (fig. 42). At approximately the same time that the primordia of the perianth set commence their development, those of the carpels are initiated, the latter as crescentic ridges arising from the inner margin of the slightly concave floral axis. Figures 48-52 show successive stages from this point extending over a period of eight to ten days. From the appearance of the carpellary primordia, there is a noticeable growth not only in length of the perianth and carpel



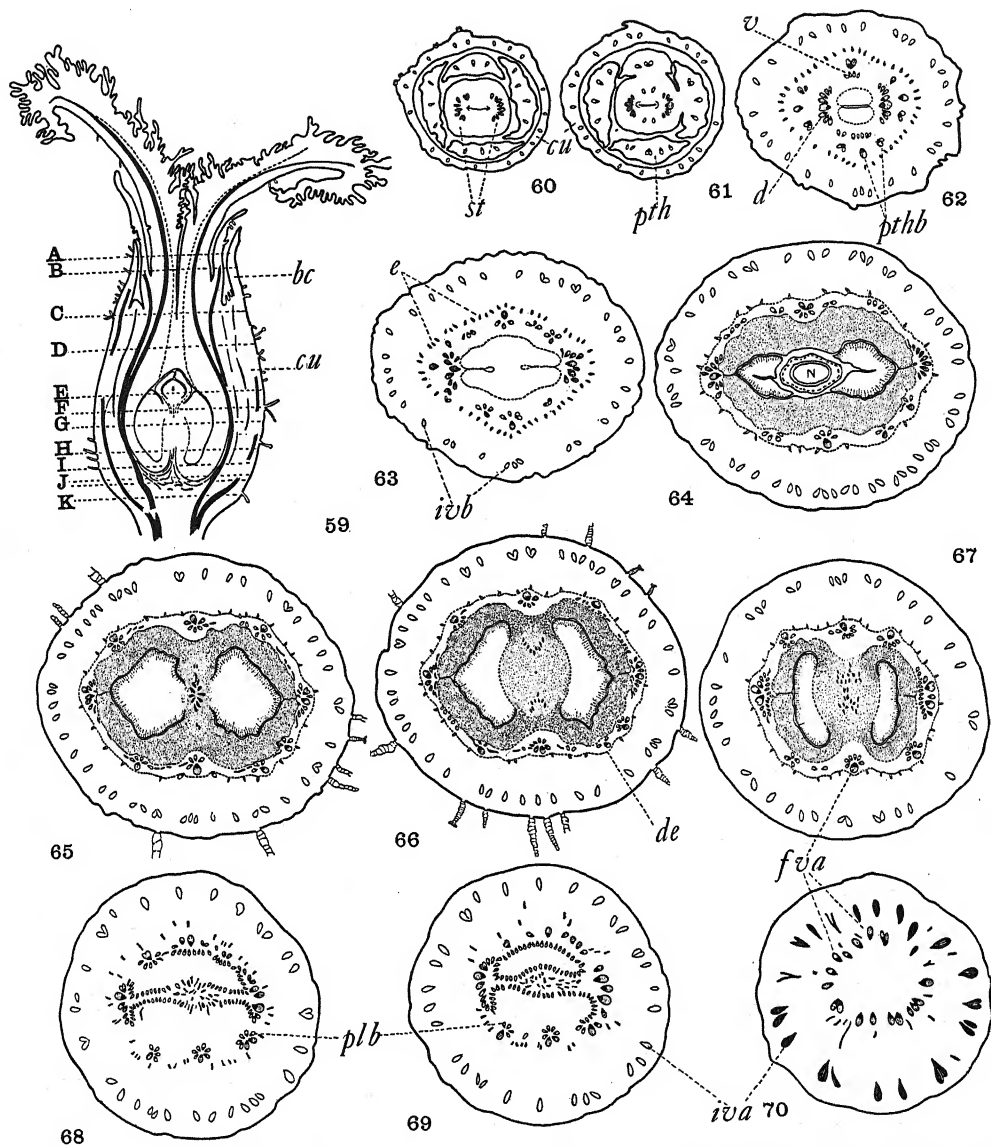
FIGS. 48-58.—Pistillate flowers and fruit of *J. mandchurica*. Figs. 48-53, median longitudinal sections of young pistillate flowers at different stages. Fig. 54, transverse section of female flower showing arrangement of parts, including staminodia. An interesting variation in another structure is here illustrated, in 2-, 3-, or 4-celled anthers all appearing in a floret. Fig. 55, flower at anthesis with ovule in megaspore stage. Fig. 56, flower at pollination time, ovule with young megagametophyte. Fig. 57, young fruit. Fig. 58, median longitudinal section of partially matured nut, closely invested by fleshy involucre, same as in fig. 46 (bc, involucral bracts; c, carpels; cu, cupule; d, dorsal bundles of style and stigma; ex, exocarp (shell); h, husk (involucre axis); fva, bundles of floral vascular axis; iva, of vascular axis of involucre; ov, ovule; pl, placental axis; pth, perianth; pthb, perianth bundle; sb, septa bundles; w, winglike parenchymatous outgrowths from placental axis which completely fill loculi prior to fruit development).

lobes but also in the axial tissue underlying these floral parts, and coincident with this a broadening and deepening of the involucrel axis. The simultaneous development of conjoined involucrel and floral axes results in the elevation of a broad floral tube bearing on its rim involucrel and perianth leaves, as well as styler components. The line of union of floral and involucrel axes is clearly distinguishable even from the earliest stages in floral development, and through the phases of maturation of the fruit is to be identified as a thin parenchymatous zone marking the line of dehiscence of involucrel husk from the ovary wall (shell).

In the meanwhile, a revival of meristematic activity at the apex of the floral axis produces a conelike structure lying between and at the base of the carpels. Stages in the differentiation of the placental column and of the ovule primordium terminating this axis are illustrated in figures 51-53. Shortly before pollination in *Juglans mandchurica*, the single integument has about closed around the nucellus (fig. 55); at pollination ovule development is completed (fig. 56) and the nucellar region contains a young megagametophyte. Early in its development the expanding placental axis meets and apparently grows conjointly with the inturred margins of the two carpels, forming a vertical partition, in the plane of the carpels, which is partly axial and partly carpellary in origin.

Even in early stages of development of the placental axis, procambium can be seen to be differentiating toward the base of the placental column from each of the eight primary groups of bundles constituting the vascular cylinder of the flower, which is here distinguished from that of the involucre, the two sets connecting independently with the stem-stele (fig. 70). Continued growth of the floral axis below the point of divergence of the placental strands gives them at later developmental stages the appearance of diverging from the bundles of the floral axis at a point midway in the ovary wall (figs. 53, 55).

More advanced stages in floral development (figs. 55-56, 59-70) indicate that the placental bundles are divergent from those of the floral stele at a level slightly higher than that from which perianth and styler bundles take their departure, and that they extend inward and—as reversely oriented bundles—downward to the base of



FIGS. 59-70.—*J. mandchurica*. Fig. 59, semi-diagrammatic longisection of mature pistillate flower showing vasculature. Figs. 60-70, structure in transverse sections at levels A-K of fig. 59 (cu, cupule; d, dorsal bundles to styles and stigmas; de, parenchymatous tissue at line of separation of ovary wall and involucre axis; e, small inversely oriented bundles at border of involucre; fva, bundles of floral vascular axis; iva, bundles of involucre axis; plb, small inversely oriented bundles derived from those of floral vascular cylinder and directed toward placental axis (pth, perianth; pthb, perianth traces; st, styles; v, lateral styler bundles, many of which exhibit reversed orientation of elements).

the cup-shaped receptacle. At this point they curve toward the center, and as a thick cylinder of bundles, somewhat confused in orientation, extend upward into the placental axis (figs. 67, 68). Not far from the base of this axis they separate as two groups of bundles, one to either side of the primary septum (fig. 66), finally converging in the vascular supply to the single ovule (fig. 65). This distribution of the placental vascular tissue apparently to two placentae has been interpreted as suggesting an ancestral, 2-ovuled condition in the flowers of this group. VAN TIEGHEM (43) and BENSON (8) placed particular emphasis upon the evolutionary significance of this feature, and BENSON furnishes a series of diagrams of the female flower of *J. regia* intended to illustrate the greater development of one of the two so-called parietal placentae. A careful study of many series of sections of the pistillate flowers and fruits of *J. mandchurica* has failed to reveal any irregularity of development of the vascular tissue distributed to either side of the placental axis, and it is suggested that atypical cases may have been examined and illustrated in these earlier papers. Also the relationship between the cupular and perianth vascular supply illustrated in BENSON's diagrams of *J. regia* bears a closer resemblance to the situation observed in *Carya* than that found in *J. mandchurica*.

Most of the space within the ovary of the walnut not occupied by the primary septum and the ovule is filled with soft parenchymatous tissue. Unlike that of the hickory (27), the packing tissue in the ovarian cavity of *Juglans* takes its origin as winglike outgrowths from the placental axis, developing at right angles to the plane of the primary septum (figs. 43, 53, 55, 56). Shortly before pollination, and for an interval of several days following, this tissue exists in a fairly compact state, completely filling the loculi and crowding close about the base of the ovule; but with growth of the ovule and later expansion of the seed coat, it is forced from the vicinity of the ovule, gradually becoming broken and chaffy in character (figs. 44, 57), except in areas of development of the secondary septa.

Developmental and anatomical features of the pistillate flower and fruit of *Carya*, essentially similar to those of *Hicoria pecan* described by SHUHART (37), show certain resemblances to and a few marked differences from those of the walnut. The resemblances are

found in the character of the ovary and its placentation, as also the manner of origin of the single orthotropous ovule. Such differences as occur are chiefly in vascular organization and evidently occasioned by reductions and compressions not found in the walnut.

Serial transections of young fruits of the hickory show outer and inner rings of vascular bundles within the fleshy involucre section, arranged in four segments corresponding to the four sectors of the cupular involucre. As in the pecan, four parenchymatous rays continuous with the edges of the involucre and perianth leaves separate the four segments of bundles, and are continuous with a zone of thin walled parenchyma marking the line of dehiscence of the cupular husk from the shell of the nut. The bundles of the inner ring of each of the four segments all show a reversed orientation of the vascular elements, and appear to be derived from the outer toral bundles by an incurving of these bundles at a point in the involucre axis slightly above the levels of divergence of the vascular elements to the perianth and involucre leaves. These reversely oriented bundles extend downward along the border of the ovary to the proximal portion of the cupule, at this point curving inward and then upward within the placental axis as two sets—one to either side of the primary septum. In *Carya*, the 3-leaved involucre and greatly reduced 1-2-leaved perianth are apparently supplied by a common, variously oriented stele, rather than by separate vascular axes as in *Juglans*. The vascular strands to the stigma tips of *Carya* are diverged from the middle inversely oriented ring of that stele, while the so-called dorsal bundles of the carpels are diverged from the center normally oriented ring. There are no vascular elements in the flower or fruit of *J. mandchurica* exactly corresponding to the dorsal carpel bundles in the hickory and pecan. The main bundles extending to the tips of the stigmas in *Juglans* are normally oriented and are diverged from those bundles of the floral vascular axis which border the midrib portion of each carpel.

Both in *Juglans* and *Carya* the sutures in the ovary wall corresponding to the lines of dehiscence of the shell bisect each carpel longitudinally. During maturation of the fruit of the walnut, lignification of the ovary wall commences with the inner section of the exocarp, extending thence to the outer border of the pericarp and

even to the axial portion of the ovary wall. Thus all eight sets of bundles constituting the floral vascular axis, as also the dorsal and lateral bundle groups to the stigmas, are included in the shell. This fact may not be apparent in figures 46 or 58, since they both depict early stages in the differentiation of the shell.

### Summary

1. In both the Fagaceae and the Juglandaceae the ovary develops as a fleshy, urn-shaped tube (the product of conjoint growth of stem and carpels), bearing on its rim perianth and styler components. A cauline placental axis growing conjointly with the in-turned carpel margins produces the septa of an ovary, which is incompletely triloculate in the Fagaceae and biloculate in the Juglandaceae.

2. The involucre is cupular and consists largely of fleshy axis. In the Fagaceae the involucre incompletely incloses the ovary as a ringlike swelling beneath the flower, bearing numerous acropetally developed scales; in the Juglandaceae it completely incloses the ovary and is united with it, forming the fibrous-fleshy indehiscent husk of the walnut and the 4-valved dehiscent husk of the hickory nut.

3. The persistence in the Fagaceae-Juglandaceae series of inner and outer fleshy regions alternating with a stony layer in the seed-incasing body suggests a pattern well defined in the gymnosperms, even to the cupule-inclosed seeds of the pteridosperms, except that both protective and nutritive functions have been to a large extent transferred from a highly differentiated seed coat to the ovary wall and involucreal structures. The outer fleshy layer (in the Fagaceae and Juglandaceae, involucreal axis) may or may not be united with the stony section of the ovary, while the inner fleshy region (endocarp) eventually becomes dry and chaffy, and in the walnuts almost indistinguishable.

4. A marked reduction in the number of foliar appendages borne by the stem axis beneath or immediately adjacent to the flower as well as a closer association of such as do appear, in combination with other specialized characters (for example, reduced number of carpels

and ovules), point to a more advanced position for the Juglandaceae than for the Fagaceae.

5. On the other hand, fundamental resemblances in the floral structure of these families, including the catkin arrangement of unisexual flowers; the axial character of ovary wall and placentation; and the major role played by the involucrel encasement of the flowers and fruits, argue in favor of their somewhat close alignment in any phylogenetic arrangement of the flowering plants.

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# CONDUCTION OF RAINFALL BY PLANT STEMS IN A TROPICAL RAIN FOREST

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 505

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(WITH TWO FIGURES)

## Introduction

Filmy ferns, bryophytes, and lichens are strikingly scarce on the trunks of many smooth-barked trees found on Barro Colorado Island, Canal Zone. This island is located in the Atlantic watershed of the isthmus in a tropical rain forest region characterized by an annual precipitation of more than 100 inches. The experiment reported here was conducted in and near the clearing occupied by the laboratory of the Institute for Research in Tropical America. A complete explanation of the paucity of epiphytes on some of the trees as compared with the abundance on others would necessitate a series of experiments before the role of various biotic as well as external environmental factors could be determined with accuracy. Time permitted the accumulation of data on only one of the possible factors involved, namely, the availability of water on the stems of woody plants during a portion of the so-called rainy season.

That a portion of every normal rain is prevented from reaching the soil by being intercepted, in the form of surface films and drops, by vegetation is well known. Some investigations on the interception of rain by foliage mention the precipitation which penetrates the canopy in reaching the ground, but do not report water flowing along branches and stems (4, 11, 14, 17). That some of the water, at least during heavier rains, may reach the ground by flowing down the trunks of trees is stated by BEALL (1), BURGER (3), McMUNN (10), and SIMSON (16).

In 1870, NEY (13) began measurements of the water flowing down the stems of forest trees. In comparing two rains which amounted to 3.39 and 7.00 mm., NEY found, respectively, 4.8 and 20 per cent of the rain in the open flowing down stems. EBERMAYER (5) described the methods used by NEY. RIEGLER (15) reports that MATHIEU

used a receptacle large enough to collect water which flowed along the stem and all that penetrated the canopy. HORTON (7) calculated from RIEGLER'S (15) records that as much as 12 per cent of the total rain flows down the trunks of beeches while as little as 1.4 per cent reaches the ground along spruce stems; 1200 liters of water ran down a beech trunk during a rain of 77.5 mm. HOPPE (6) recorded the rainfall intercepted by rain gages placed under the canopy of forest trees in central Europe. By means of troughs placed around the trunks of trees in the same plot he recorded the amount of stem flow. Thus he was able to compute the amount of water actually retained by the tree crowns, which constitutes a loss to the forest soil. ZON (19, p. 26) states that in a conifer forest 0.7 to 3.0 per cent of the total precipitation reaches the ground along branches and trunks, but in a deciduous forest 15 per cent is conducted in this manner. MUNNS and SIMS (12) report that 1-5 per cent of the total precipitation reaches the ground by running down the stems of pine trees in the Appalachian region. BROOKS (2) recorded data on the rainfall distribution under *Cryptomeria japonica* as reported by OVSYANNIKOFF. The writer's calculations show that 1.6 to 14.6 per cent of the rainfall in the open may trickle down the trunks.

HORTON (7) diverted stem-flowing water into large containers by means of lead troughs fitted around the trunks of deciduous and hemlock trees in New York. The maximum amount collected from beech trees was 9.9 per cent of the total rainfall. He concludes that trees with rough bark conduct the least water to the ground. At present researches are being conducted on rainfall interception by trees in various parts of the United States.<sup>1</sup>

Except for the researches of RIEGLER (15), the results of the investigations just discussed are expressed in depth of water on the basis of crown area. The water returning to the ground along stems of plants on Barro Colorado Island has been calculated on the basis of depth of water in the cup and expressed as percentage of the total rainfall measured in the forest clearing.

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FIG. 1.—Apparatus used in collecting water draining down stems of plants. *A*: leafless control stem (station I) with *Nothopanax guilfoylei* hedge and stations IV and V in middle left. *B*: upper smaller cup (station IV) and lower larger cup (station V) on *Nothopanax*.

### Material and methods

A rain gage of the standard tipping bucket type, as described by KADEL (9), has been in operation on Barro Colorado Island for several years and served to determine the rainfall in the laboratory clearing for the period of this experiment. The electrically recorded data as well as direct measurements of the rainfall are a part of the scientific records of the island.

Plants utilized in this study were growing under cultivation or naturally near the laboratory. Only five stations with a total of five collecting cups were used, as follows:

A small unidentified tree was stripped of leaves and branches and placed in the ground at a location where the prevailing northeast winds would not be obstructed by buildings or vegetation. This 6-foot naked stem served as a control for the experiment (fig. 1A).

A young soursop or guanábana tree, *Annona muricata* L., growing under cultivation near the control station was selected because it was isolated except for the nearby canopy of the 75-foot trees at the edge of the clearing. This tree was in a vigorous vegetative condition and about 12 feet in height (fig. 2A).

A 9-foot high urticaceous tree, *Myriocarpa yzabalensis* (Donn. Smith) Killip, growing in the dense rain forest was selected for suitable trunk diameter and accessibility. Although this species is commonly considered a shrub, this specimen possessed a solitary trunk. The plant leaned from the vertical about 20°, and possessed few leaves. The latter condition is common for such understory plants (fig. 2B).

A single vertical branch of the shrub *Nothopanax guilfoylei* (Cogn. and Marchal) Merrill, an introduced species, supported two cups in tandem (fig. 1A, B). The ornamental hedge of which this plant was a part was trimmed about once a month and possessed terminal tufts of leaves. Occasionally a small group of compound leaves grew from short branches along the trunk. All stations were approximately 212 feet above sea level.

Rainwater flowing down the stems of these plants was collected in wide-angled disks or cups constructed of tin. The sides of the cups



FIG. 2.—Apparatus used in collecting water flowing along stem of two tropical trees. A: *Annona muricata* in laboratory clearing with rain forest in background (station II). B: *Myriocarpa yzabalensis* growing in rain forest (station III).

formed an angle of about  $65^{\circ}$  with the sides of the stem. Cup dimensions are given in table 1.

A strip of rubber automobile inner tube, 2 inches wide and long enough to overlap slightly, was wrapped tightly around the plant at the desired height and the overlapping ends securely fastened with liquid rubber cement. Around this rubber collar the tin cup was modeled and soldered. Since all plant stems increase in circum-

TABLE 1

STATION	LOCALITY	NAME OF PLANT AND TOTAL HEIGHT (IN.)	CIRCUM- FERENCE OF STEM AT CUP LEVEL (IN.)	CUP			
				HEIGHT ABOVE GROUND (IN.)	CIRCUM- FER- ENCE (IN.)	DEPTH (IN.)	NET AREA (SQ. IN.)
I.....	About 48 ft. from rain gage*	Leafless trunk of unknown species, 70	3.82	31	17.48	1.63	23.16
II.....	About 30 ft. beyond sta- tion I	<i>Annona muri- cata</i> , 144	3.94	50	17.48	1.63	23.08
III.....	About 70 ft. beyond sta- tion II	<i>Myriocarpa yzabalensis</i> , 112	3.82	24	17.48	1.63	23.16
IV.....	About 40 ft. from rain gage (near station I)	<i>Nothopanax guilfoylei</i> , 63	2.48	22.5	8.82	0.52	5.70
V.....	Same as sta- tion IV	Same as sta- tion IV	2.64	13	17.48	1.63	23.76

\* Rain gage 12 feet from library.

ference downward, a downward pressure on the cup resulted in a tighter fit against the rubber cylinder and prevented leakage. As an added precaution against water loss, the inside of the cup and the stem immediately above it were coated with liquid rubber cement.

A 2-inch length of  $\frac{1}{4}$ -inch copper tubing was soldered into the side of each cup, approximately halfway between the rim and the base. Water accumulating in the cup rose to the level of the tube and ran down it and connecting rubber tubing into a suitable glass container. Insertion of the tube at some distance from the bottom of the cup prevented the daily accumulation of insects from obstructing the orifice. Such debris usually sank to the bottom of the cup. The

water in the container at the base of the plant as well as the water remaining in the cup was collected and measured every 12 hours, at 6 A.M. and 6 P.M.

Since the object of this experiment was to determine the relative amount of water available on the stem and branches as a possible factor in the development of epiphytes, water flowing down the stems was calculated in inches based on the net cup area. In table 2 such data are expressed in percentages of the rainfall measured by the rain gage.

### Observations

The official rain gage was permanently installed in the laboratory clearing far enough from laboratory buildings and trees so that no interference was observable. Water collected in the cup on the control stem (station I) represents the accumulation of rain striking the cup directly and of water intercepted by the stem above the cup.

The *Annona* tree in the clearing possessed a well developed crown and was in full foliage. Repeated observations during storms showed that only an occasional drop of water filtered through the canopy and fell into the cup, which was placed on the trunk just below the divergence of the lowest branch from the trunk. Investigations by McMUNN (10) and HOPPE (6, p. 24) show that the crown of a tree permits the least penetration of water nearest the trunk. Since this *Annona* tree was very near the edge of the laboratory clearing, some water during a calm rain may have dripped from the 75-foot canopy of the rain forest trees, striking the foliage of the experimental tree. During rains accompanied by considerable wind the prevailing direction of the rain precluded such a dripping of water from the nearby forest. All water collected in the cup of station II, therefore, was that which ran down the leaves and along smaller branches until it finally flowed down the main stem.

The *Myriocarpa* tree in the rain forest near the laboratory possessed only a small number of leaves and was leaning from the vertical sufficiently so that most of the water flowing down its trunk was that which dripped from leaves and branches in the canopy of the main forest. It represents an average situation for many of the understory trees in such a forest where the canopy is 75-100 feet above the forest floor (station III).

The hedge plants of *Nothopanax* bordered an unobstructed path near the control stem (station I) and were nearer the official rain gage than the other experimental plants. Characteristically *Nothopanax* plants possess a main trunk only a few inches in length from which several nearly perpendicular branches originate. These branches are nearly leafless for a distance of about a yard and are repeatedly pruned at the top so that the terminal tuft of leaves originates from several axillary branches. A small cup was placed on a branch just under such a tuft of leaves so that very little rain could strike the cup directly. Most of the water accumulating in the cup, therefore, had been intercepted by the leaflets and had drained down the rachises, petioles, smaller branches, and the branch around which the cup was fastened (station IV).

A cup as large as those installed in stations I, II, and III was placed on the same *Nothopanax* stem about 9.5 inches below the smaller cup. Immediately above the large cup a short branch with three well developed compound leaves was growing from the vertical branch. Water accumulating in this cup had two origins: rain striking the cup on the windward side and water intercepted by a tuft of leaves lateral to the stem. This is designated as station V.

Only the volumes of water collected twice daily and the necessary dimensional data on the apparatus and plants utilized in the experiment were recorded on Barro Colorado Island. Rainfall in inches is recorded only for the rain gage. Records from all the experimental stations were converted into percentages of the officially recorded rainfall. The percentages are based on rainfall in inches of the net areas of the cups. Table 2 summarizes the results of the experiment.

The 14.01 inches of rainfall of August, 1938, was more nearly normal than the 19.36 inches of August, 1937, and approached to within 0.5 inch the amount recorded for August, 1936 (18). Of the 14.01 inches of rainfall recorded in August, 1938, 8.68 inches (62.0 per cent) fell in the 13 days covered in this experiment; therefore the amount of rain during this period was slightly above average. Table 2 gives the rainfall by 12-hour periods from August 5 to 18. During this period 1.70 inches fell in the night periods and 6.98 inches (80.4 per cent) in the daylight hours. Heavy rains taxed the capacity of the collecting containers, which at times were too small.

No records were taken when any doubt existed about the reliability of the measurements. With the high relative humidity of a tropical rain forest region it was possible to find traces of water in the cups after rains which yielded only 0.01 and 0.02 inches of water as measured by the official rain gage. While a rain of 0.04 inches was suf-

TABLE 2

AUGUST, 1938 (HALF-DAYS)	RAINFALL (IN.)	PERCENTAGES OF RAINFALL				
		I. CONTROL (BARE STEM)	II. ANNONA	III. MYRIO- CARPA	NOTHOPANAX	
					IV. UPPER	V. LOWER
5-6.....	0.19	94.2	659.5	526.9	157.9	57.9
6.....	0.01	Trace	Trace	Trace	Trace	Trace
6-7.....	0.19	91.6	.....	798.9	95.8	61.0
7.....	0.54	102.4	563.1	.....	148.7	39.4
7-8.....	0.04	80.0	717.5	1027.5	280.0	32.5
8.....	0.16	96.9	388.1	512.5	86.9	61.3
8-9.....	0.41	99.0	622.2	573.9	348.5	24.1
9.....	0.22	92.7	310.5	682.7	330.9	33.6
9-10.....	0.13	101.5	1151.5	1103.1	411.5	30.0
10.....	1.71	89.2	.....	.....	289.2	26.7
10-11.....	0.32	102.2	1141.8	805.3	401.2	20.0
11-13.....	No rain for 5 half-days					
13-14.....	0.13	93.1	607.7	634.6	246.9	31.5
14.....	0.60	103.7	669.0	699.7	271.2	36.0
14-15.....	0.01	Trace	Trace	Trace	Trace	Trace
15.....	0.86	95.9	.....	.....	311.2	34.3
15-16.....	0.26	95.4	.....	.....	309.0	34.6
16.....	2.38	104.2	540.3	559.4	305.8	16.6
16-17.....	0.02	Trace	Trace	Trace	Trace	Trace
17.....	0.02	Trace	Trace	Trace	Trace	Trace
17-18.....	0.00	0	0	0	0	0
18.....	0.48	103.1	633.5	620.4	263.1	36.4
Average.....	.....	96.6	667.1	712.1	266.1	36.0

ficient to cause an almost normal accumulation of water in the cups (August 7-8), NEY (13) reports that in European forests more than 2 mm. (0.08 inch) of rain must fall before water runs along the stems. At times evaporation losses were negligible because of the high relative humidity and the frequent and regular collection periods.

The cup on the leafless control stem collected nearly as much and even slightly more water than the rain gage. The lowest percentage (80 per cent for August 7-8) was recorded for a rain which fell at the

beginning of the collection period. The highest percentage (104.2 per cent for August 16) was recorded for the heaviest rain of the entire month. During a 2-hour period on August 16 a total of 2.21 inches of rain fell, which is 15.8 per cent of the total for the month. The average percentage of water collected by the control cup (96.6 per cent) in terms of the water collected by the rain gage is equal to the fidelity of rain gages placed some distance apart in areas of less rainfall (8).

Water accumulation in the cups on trees in stations II and III was very great. The least water collected from the *Annona* tree was 310.5 per cent (August 9) and from the *Myriocarpa* tree 512.5 per cent (August 8), while the most water collected from these trees was 1151.5 and 1103.1 per cent, respectively (August 9-10).

Most of the variations in the percentage of water collected at any one station may be attributed to differences in the intensity, direction, and time of the rain with reference to the collection periods. The maximum percentage of water collected by stations II and III was during the night periods (August 7-8, 9-10, and 10-11), when the total precipitation as recorded by the rain gage was relatively small (0.04, 0.13, and 0.32 inches, respectively). Rain during these periods fell either at the beginning of the collection period (6 P.M.) or at irregular intervals during the night, so that in either case a very high relative humidity was assured. During such periods water dripped from leaf to leaf and branch to branch, reducing the evaporation losses.

The average percentage for the *Annona* tree station of 667.1 approaches the 712.1 per cent average of the *Myriocarpa* tree station, even though the two stations were some distance apart, one in the open and the other in the rain forest itself.

The *Nothopanax* shrub (station IV), with a small terminal tuft of leaves, did not intercept and conduct via the stem nearly as much water as did the larger *Annona* tree or the canopy-covered *Myriocarpa* tree. With percentages of rainfall ranging from 86.9 to 411.5, it is evident that the maximum percentage recorded barely exceeds the minimum percentage intercepted and conducted by the *Annona* tree. Since the circumference of the cup at this station was much smaller than the others, rain striking it directly seems to be negli-

gible. The smaller percentage of water collected may be attributed to the small canopy of the plant.

The last (station V) was a large cup on the same *Nothopanax* stem and about 9.5 inches below station IV. Even though this cup was equal in size to the cups in stations I, II, and III, and was more exposed to direct rain than any other cup except the control, very little water accumulated. Other than direct collection of rain, the sources of water would be the 9.5 inches of stem between the upper and the lower cups and the three compound leaves growing from a small lateral branch immediately above this lower cup. The minimum percentage of water collected from station V was 16.6 of the total for that period and the maximum was 61.3. Both records are from daylight periods.

The leafless control stem was nearly devoid of lichens and possessed no other visible epiphytes.

The *Annona* and the *Myriocarpa* trees possessed a few non-fruiting crustose lichens and only scattered and poorly developed mats of clinging leafy hepatics.

Plants of *Nothopanax*, which are terminally pruned, often are infested with termites. The loose bark of such plants supports a luxuriant growth of leafy liverworts. Even where no termites have invaded the stem, large clusters of such hepatics grow where dripping water strikes a local area. These *Nothopanax* plants support a copious growth of leafy hepatics even though their habitat assures greater exposure and lower relative humidities during daylight hours.

### Summary and conclusions

1. Water draining down stems of woody plants growing on Barro Colorado Island, Canal Zone, was collected by means of cups and measured. The amount of water collected was calculated to inches of water on the basis of the net area of the cup and expressed as percentage of the rainwater collected by the official rain gage. The experiment covered a period of 13 days in August, 1938.

2. A leafless control stem, equipped with a collecting cup, collected 80.0 to 104.2 per cent as much water as the rain gage.

3. Cups installed on the stems of *Annona* and *Myriocarpa* trees,

the former in the laboratory clearing and the latter in the rain forest, collected six to seven times more water than the rain gage.

4. Cups arranged in tandem on a stem of *Nothopanax* collected an amount of water consistent with their position. The smaller upper cup, which caught the water flowing down the leaves and smaller branches of the trimmed crown, collected an average of two and one-half times as much water as recorded by the rain gage. The lower larger cup, into which water from a short portion of the stem and from a lateral group of leaves drained, collected on the average slightly more than a third as much water as the rain gage. These *Nothopanax* plants possessed well developed mats of leafy liverworts and lichens.

5. From the results it seems conclusive that during the rainy season in a tropical rain forest area an amount of water flows down the stems of woody plants sufficient to support a copious growth of epiphytes such as lichens, liverworts, mosses, and filmy ferns.

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## PHOTOPERIODIC RESPONSES OF SEVERAL VARIETIES OF SOYBEANS

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(WITH TEN FIGURES)

### Introduction

Previous work on the Biloxi soybean (3, 8) indicates that a succession of at least two relatively short photoperiods, each followed by a relatively long dark period, is necessary for photoperiodic induction in that plant. Such a succession of light and dark periods does not seem to be essential in all short-day plants, since in *Xanthium* (5) photoperiodic induction may follow exposure to a single relatively long dark period, whether the photoperiods are relatively long or short. The present studies report additional data concerning the photoperiodic response of several varieties of soybeans.

GARNER and ALLARD (4) have shown that soybean varieties planted during the summer months differ greatly in their time of bloom. They found that the variety Mandarin produced open flowers as quickly during the summer as during the winter months; other varieties, such as Biloxi and Peking, required many more weeks during the summer than during the winter. These differences, they concluded, were caused primarily by the different photoperiodic requirements of the several varieties.

From the work of GARNER and ALLARD it is evident that photoperiodic conditions appropriate to cause suppression of flowering in the Mandarin variety were not attained even during the summer when the length of effective photoperiod probably exceeded 15 hours. Since the range of photoperiods under which this plant would flower was obviously wide, it seemed possible that varieties might be found that could initiate flower primordia on long daily photoperiods or even in continuous illumination. Studies were made of Mandarin and several other varieties to determine their responses under extremely long photoperiods and under continuous illumination.

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For the studies dealing with floral initiation and growth, two experiments were carried out, one in the fall of 1938 and one in the spring of 1939, to determine the critical photoperiod for initiation in each of several varieties. Certain leaf areas were determined and stem-length measurements were taken. Data were also collected concerning flowering and fruiting under the various photoperiods employed. Two less extensive experiments, each involving a single variety, were carried out to obtain more detailed information concerning the influence of length of photoperiod upon the time and place of initiation of flower primordia. A chemical study was made of two varieties that differed greatly in their ability to initiate flower primordia under conditions of long photoperiod.

### Material and methods

The varieties chosen for the experiments were selected on the basis of the average time required from planting to maturity when

TABLE 1  
AVERAGE NUMBER OF DAYS FROM PLANTING TO MATURITY  
OF VARIETIES OF SOYBEANS

VARIETY	DAYS TO MATURITY*	VARIETY	DAYS TO MATURITY
Otootan.....	175	Ito San.....	105
Avoyelles.....	170	Minsoy.....	100
Biloxi.....	165	Wisconsin Early Black...	100
Tokyo.....	140	Mandarin.....	100
Peking.....	125	Mc Crostie's Mandarin .....	
Mandell.....	110	Batorawka.....	
Hudson Manchu.....		Agate.....	90

\* Data from MORSE and CARTER (6).

grown under field conditions. They ranged in this requirement from 90 to 175 days (table 1), according to the data of MORSE and CARTER (6).

Plants for all studies of floral induction were grown in 4-inch pots in the greenhouse. They were kept on warehouse trucks and were moved into adjoining dark chambers at the close of their respective photoperiods. The photoperiods for all lots receiving daily alternations of light and dark were begun at 8 A.M., and consisted of nat-

ural illumination extended with Mazda light of approximately 50 foot-candles for those lots receiving photoperiods longer than the natural ones.

In two experiments, twelve varieties of soybeans were each grown under nine different conditions of photoperiod. Six plants of each of the twelve varieties were placed on a truck and each truck was subjected to a different length of photoperiod. The photoperiods consisted of 8, 10, 12, 14, 16, 18, 20, 22, and 24 hours daily. Photoperiodic treatment was begun when the plants first came through the soil and was continued without interruption until they were harvested.

In other experiments involving single varieties and two or three photoperiods, approximately fifty plants were used per treatment.

#### Experimental results

The twelve varieties used in the fall experiment of 1938 were the same, with one exception, as those used in the spring experiment of 1939. The variety Agate, used in the fall experiment, was replaced in the spring by Batorawka, an early maturing variety recently introduced from Poland. Since the other eleven varieties responded similarly in both experiments, the results of only the spring experiment are presented in detail.

This experiment was started March 21, and the plants were allowed to grow until May 9 before dissections were made. At this time all the varieties had bloomed and begun to set fruit on 8- and 10-hour photoperiods, and several varieties had produced visible buds or open flowers on still longer photoperiods.

The plants that had not yet produced open flowers or visible buds were dissected on May 9, 50 days after planting. At this time flower primordia were present on every plant subjected to continuous illumination in the seven varieties: Hudson Manchu, Mandell, Minsoy, Mc Crostie's Mandarin, Batorawka, Wisconsin Early Black, and Mandarin. These varieties are all early, requiring 110 days or less from planting to maturity. In Biloxi, Otootan, and Avoyelles, no flower primordia were present on plants receiving 16-hour or longer photoperiods. These varieties require 165 or more days to reach maturity, and behave as late varieties. In Tokyo and Peking va-

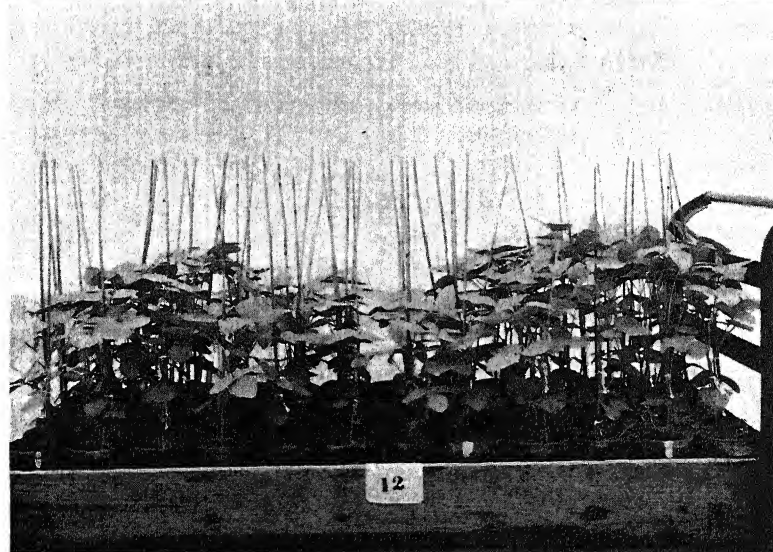
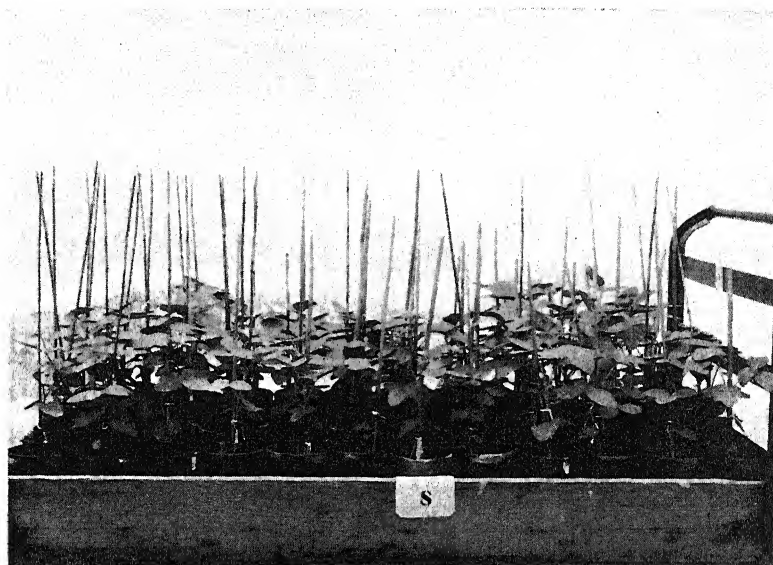


FIG. 1.—Twelve varieties of soybeans grown on 8- and 12-hour photoperiods respectively. Arranged in same order as listed in table 2; Mandarin at left and Biloxi at right. Photographed May 3, 1939, 43 days after planting.

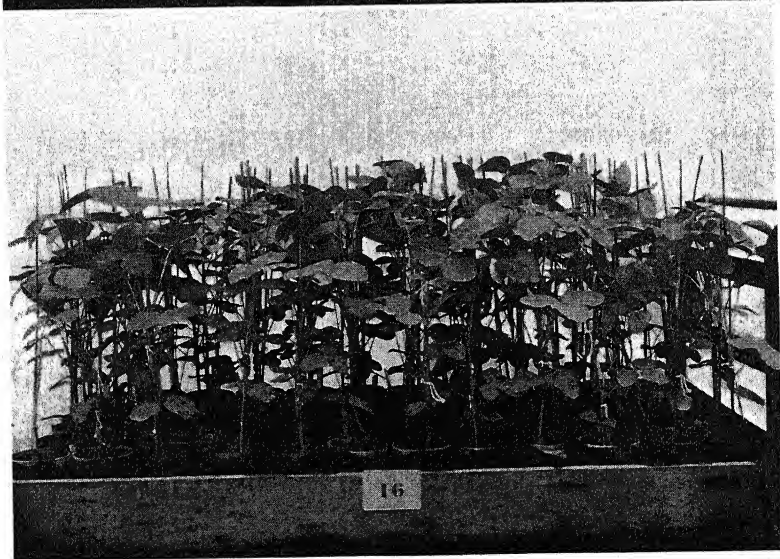
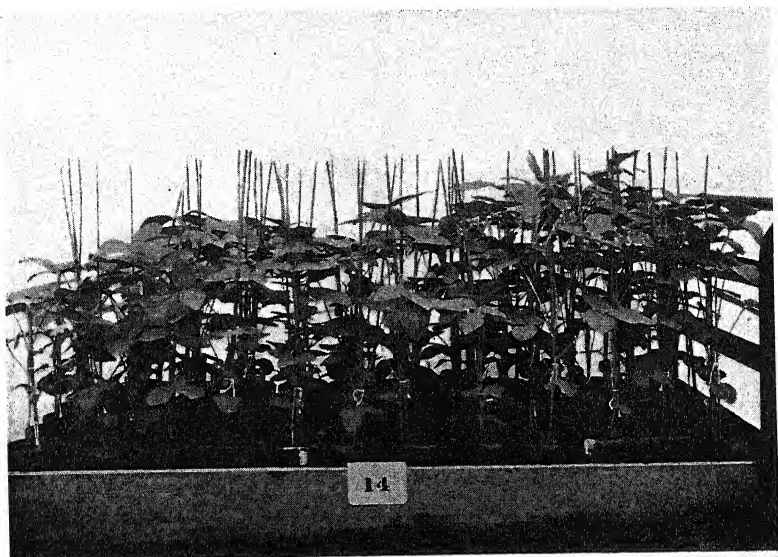


FIG. 2.—Twelve varieties of soybeans grown on 14- and 16-hour photoperiods respectively. Arranged in same order as listed in table 2; Mandarin at left and Biloxi at right. Photographed May 3, 1939, 43 days after planting.

rieties, flower primordia were present in plants receiving 16-hour photoperiods. When these two varieties were subjected to photoperiods longer than 16 hours, flower primordia were formed on occasional plants of Tokyo but on no plants of Peking. These varieties are reported as intermediate in time of maturity, Peking requiring 125 days and Tokyo 140.

The results show that there is a relationship between earliness of maturity and length of photoperiod on which flower primordia can be initiated. In general, the earlier the variety matures the longer the photoperiod on which initiation can occur. This was probably to be expected from observations based on time of flowering as influenced by photoperiod (4). Since seven of the twelve varieties investigated were capable of initiating flower primordia with continuous illumination, this fact would seem to have a direct bearing on the problem of the relation of the dark period to photoperiodic induction in such short-day plants as soybeans. The fact that floral initiation occurred in several varieties regardless of the length of photoperiod employed might suggest that these varieties are not truly short-day plants, but should be regarded as indeterminate. While these varieties produced flower primordia on continuous illumination, blossoming and fruiting did not occur on photoperiods longer than 16 hours except in the variety Batorawka. In character and height of growth they also showed differences in response to photoperiod. In the past, macroscopic observations of flower bud formation, time of flowering and fruiting, and general growth behavior have been the criteria used in most cases in classifying the responses of plants to photoperiodic treatment. From this viewpoint these varieties should be regarded as short-day plants.

In all varieties the height attained by the plants was markedly influenced by length of photoperiod, greater height being associated with longer photoperiod (figs. 1-10). Part of this difference was caused by the fact that there were actually more internodes present in the plants that received long photoperiods than in those that received short ones. Plants grown on photoperiods shorter than the critical for flowering discontinued the differentiation of new structures at their tips rather early, while this was not the case in plants grown on photoperiods longer than the critical.

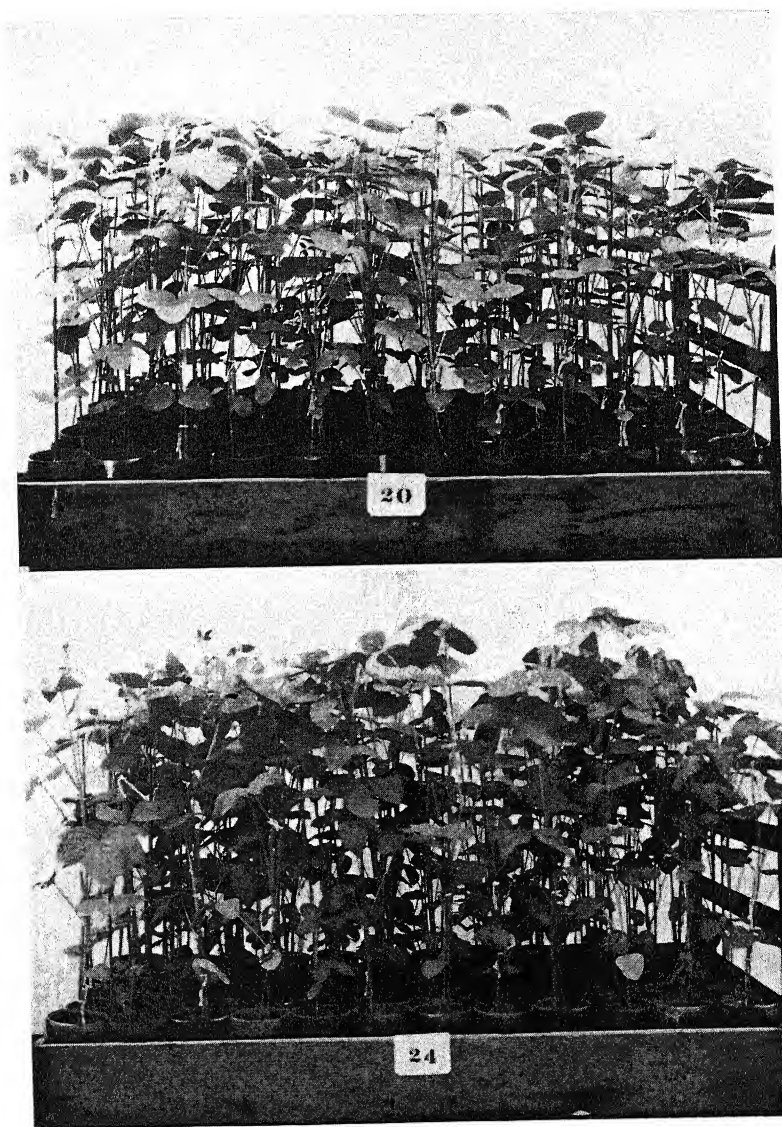


FIG. 3.—Twelve varieties of soybeans grown on 20- and 24-hour photoperiods respectively. Arranged in same order as listed in table 2; Mandarin at left and Biloxi at right. Photographed May 3, 1939, 43 days after planting.

Part of the differences in plant height were also caused by differences in the lengths attained by corresponding internodes in response to photoperiodic treatments. Such differences appeared in internodes that reached maturity very early in the life of the plants. Thus the length of the first internode was greatest in lots receiving longest photoperiods.

TABLE 2  
EFFECT OF 8-, 16-, AND 24-HOUR PHOTOPERIODS UPON  
STEM LENGTH AND LEAF AREA OF SOYBEANS.  
PLANTED MARCH 21; MEASURED MAY 4

VARIETY	MEAN LENGTH OF STEM FROM COTYLEDONS TO PRIMARY LEAVES (CM.)			MEAN AREA OF PRIMARY LEAVES (CM. <sup>2</sup> PER LEAF)		
	8-HOUR	16-HOUR	24-HOUR	8-HOUR	16-HOUR	24-HOUR
Biloxi .....	3.9	5.2	7.0	24.2	24.6	25.5
Otootan .....	2.6	2.9	5.0	12.5	12.2	11.6
Avoyelles .....	2.7	3.8	5.7	14.7	13.4	19.2
Tokyo .....	4.7	4.8	7.9	24.9	27.3	27.7
Peking .....	2.2	2.6	3.2	10.9	13.6	14.0
Hudson Manchu .....	3.9	5.1	6.3	17.9	17.1	21.4
Mandell .....	3.4	4.2	5.0	17.2	24.1	22.7
Minsoy .....	3.5	4.0	5.1	9.1	9.1	9.5
Mc Crostie's Mandarin .....	3.9	5.2	6.7	15.9	19.4	19.5
Batorawka .....	4.4	5.3	7.5	23.5	27.6	33.1
Wisconsin Early Black .....	3.4	4.7	5.6	16.6	21.3	19.3
Mandarin .....	4.9	6.2	7.7	17.6	20.2	20.8
Mean .....	3.6	4.6	6.1	17.1	19.2	20.4

The mean areas of the primary leaves of each variety were also determined. These leaves were attaining their mature size at approximately the same time that elongation was occurring in the first internode. The differences in their areas as influenced by photoperiod were not so large nor so consistent as were the differences in internode length (table 2).

Although floral initiation in seven of the twelve varieties was not inhibited by long photoperiod nor by continuous light, the development of flowers and fruits showed response to photoperiod in all varieties.

The total number of pods 2 cm. or more in length was determined

for each lot of six plants when they were 50 days old (table 3). Plants of only one variety produced pods of this size on photoperiods of 18 or more hours. All the varieties that were able to initiate flower primordia in continuous light formed one or more pods on 16-hour photoperiod. On 14-hour photoperiod a few pods were formed on

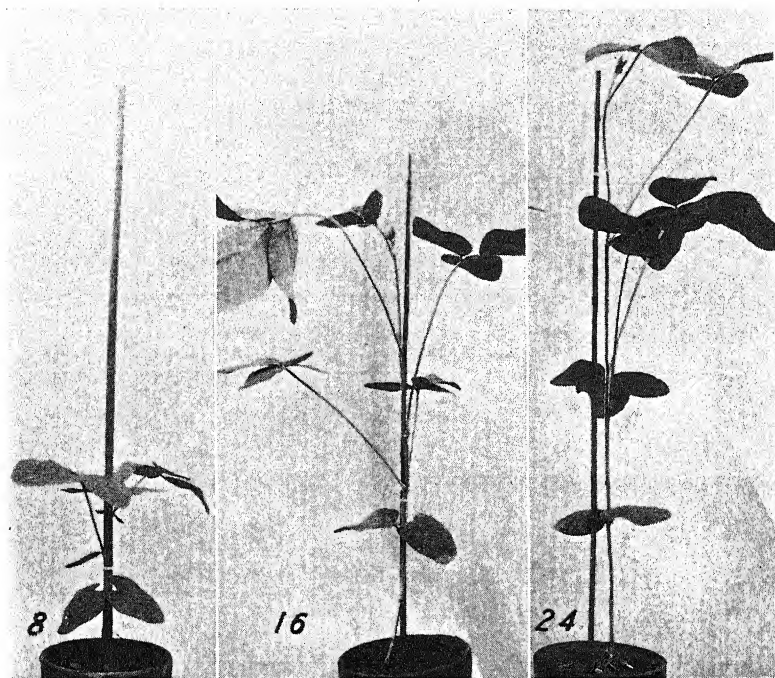


FIG. 4.—Biloxi soybeans grown on 8-, 16-, and 24-hour photoperiods respectively. Photographed May 3, 1939, 43 days after planting.

one additional variety, Peking, but none was formed on the remaining four varieties. On 12-hour and all shorter photoperiods there were one or more pods in every lot. Many pods shorter than 2 cm. were present when the data were obtained. These continued to grow and the number of large pods per lot increased. This increase occurred only in those lots of table 3 containing one or more pods and in the 14-hour lot of the variety Tokyo. The remaining lots showing no pods in table 3 did not develop any subsequently. From these re-

sults it seems evident that certain varieties can initiate flower primordia under conditions of long photoperiod at which their further development into mature fruits is inhibited.

The results obtained with Agate in the fall experiment of 1938 were similar to those obtained in the spring experiment with Batorawka. Agate plants produced visible buds and open flowers on every photoperiod used, including continuous light. Of the varieties

TABLE 3

EFFECT OF PHOTOPERIODS OF VARIOUS LENGTHS UPON PRODUCTION  
OF FLOWER PRIMORDIA, FLOWERS, AND FRUITS IN  
SOYBEANS. PLANTS 50 DAYS OLD

VARIETY	TOTAL PLANTS PER LOT OF SIX BEARING FLOWERS OR FLOWER PRIMORDIA						TOTAL PODS* PRODUCED PER LOT OF SIX PLANTS					
	14- HOUR	16- HOUR	18- HOUR	20- HOUR	22- HOUR	24- HOUR	8- HOUR	10- HOUR	12- HOUR	14- HOUR	16- HOUR	18- HOUR
Biloxi.....	6	0	0	0	0	0	28	22	1	0	0	0
Otootan.....	6	0	0	0	0	0	35	37	2	0	0	0
Avoyelles.....	6	0	0	0	0	0	40	48	16	0	0	0
Tokyo.....	6	6	3	0	1	1	35	36	22	0	0	0
Peking.....	6	6	0	0	0	0	25	32	30	3	0	0
Hudson Manchu.....	6	6	6	6	6	6	20	22	19	26	15	0
Mandell.....	6	6	6	6	5	6	29	18	9	19	1	0
Minsoy.....	6	6	6	6	6	6	29	35	29	30	8	0
Mc Crostie's Mandarin.....	6	6	6	6	6	6	21	24	31	26	24	0
Wisconsin Early Black..	6	6	6	6	6	6	26	38	37	33	4	0
Mandarin.....	6	6	6	6	6	6	11	13	24	24	24	0
Batorawka.....	6	6	6	6	6	6	33	31	31	35	22	2

\* Pods 2 cm. or more long.

tested, Agate and Batorawka seemed least inhibited in their floral development by long photoperiod. Since the effect of photoperiod on the time and place of origin of the first flower primordia was not determined in either of the previous two experiments, this point was studied further with the variety Agate.

Three lots of plants were started April 23, 1939. One was placed on 8-hour photoperiod, another on 16-hour, and the third on continuous illumination, before the seedlings began to emerge from the soil. On May 6, when the plants were thinned, dissections were made of some seedlings from each treatment. Flower primordia were

present on the 8-hour plants but not on the others. Since flower primordia were found on the 8-hour lot, the plants were harvested and dissected on May 8, 15 days after planting. At this time the largest plants were expanding their first compound leaf. Flower pri-

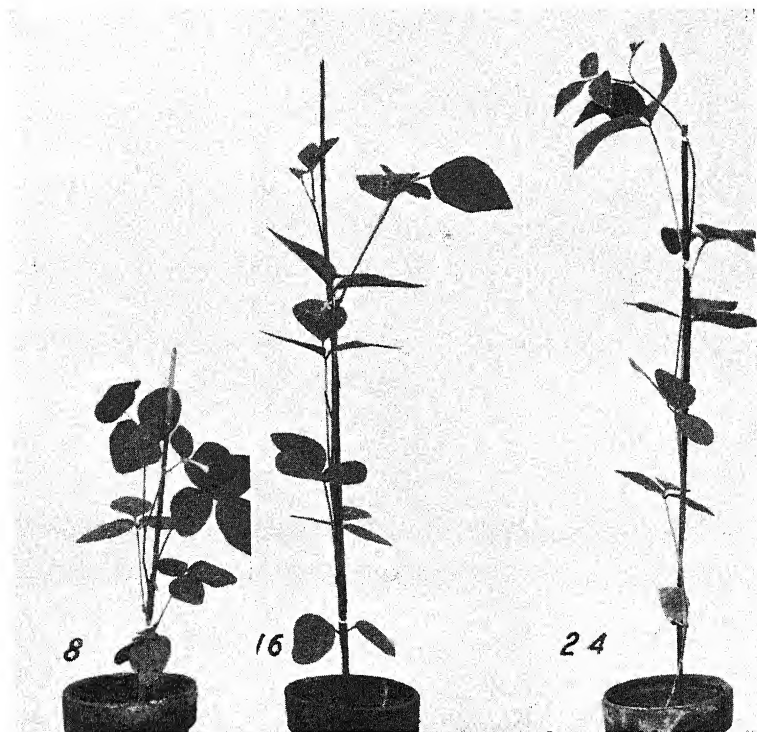


FIG. 5.—Ootootan soybeans grown on 8-, 16-, and 24-hour photoperiods respectively. Photographed May 3, 1939, 43 days after planting.

mordia were present on forty-seven of the fifty-three plants harvested. The first flowers to differentiate were, with few exceptions, in the axil of the second compound leaf.

On May 12, 19 days after planting, the other two lots of plants were also dissected. Flower primordia were present on all the plants on 16-hour photoperiod and on all but a few stunted plants of the lot receiving continuous illumination. In the 16-hour lot the first flowers were formed in the axil of the fourth compound leaf in nearly

all the fifty plants dissected. In those receiving continuous light the first flowers were formed in the axil of the fifth compound leaf in all but a few plants, in which they were formed in the axil of the sixth compound leaf.

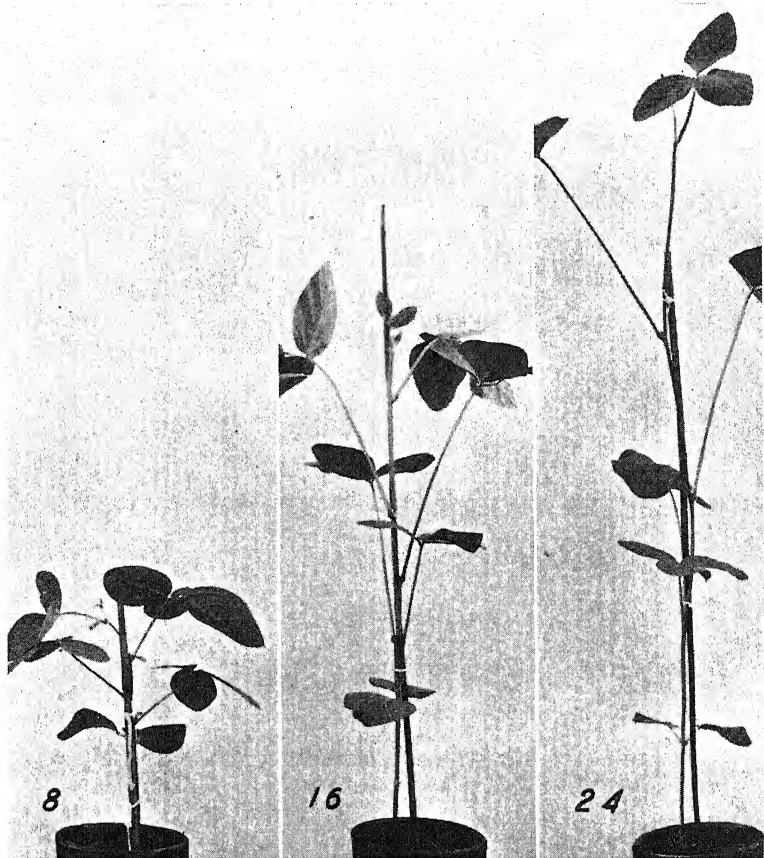


FIG. 6.—Tokyo soybeans grown on 8-, 16-, and 24-hour photoperiods respectively. Photographed May 3, 1939, 43 days after planting.

These observations on Agate soybeans show that long photoperiods cause a retarding influence upon time of initiation of flower primordia, even in a variety that promptly initiates them under 8-hour photoperiods. They were present on plants of the 8-hour lot

in less than 2 weeks after planting, but were not present at that time on plants grown on 16-hour photoperiod or on continuous light.

The differences in position of the lowermost, and therefore first formed, flower primordia on plants of the three lots also showed that photoperiod influenced the time, and consequently the place, of origin of flower primordia. The photoperiodic stimulation resulting

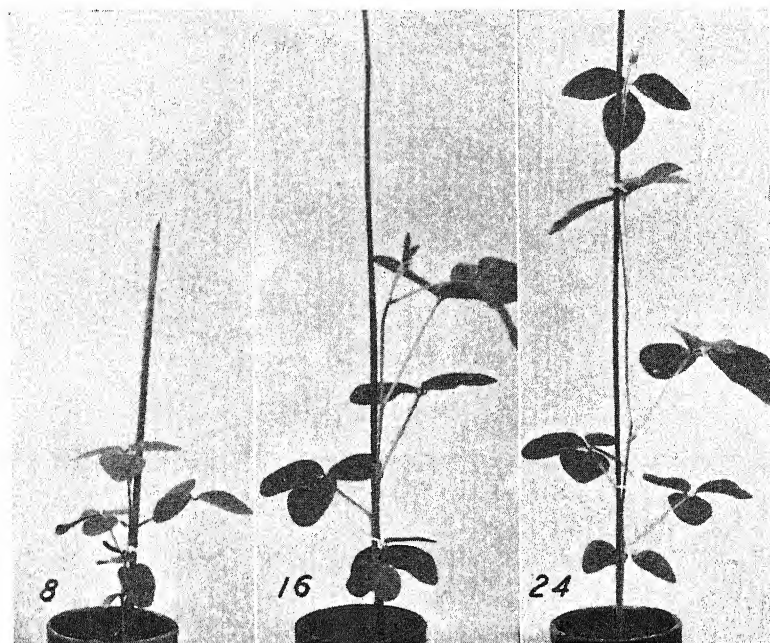


FIG. 7.—Peking soybeans grown on 8-, 16-, and 24-hour photoperiods respectively. Photographed May 3, 1939, 43 days after planting.

from treatment with 8-hour photoperiod was sufficient to bring about formation of flower primordia in the bud in the axil of the second compound leaf. This same bud in the other two lots developed into a vegetative structure, however, as did also the next one above it. Evidently the photoperiodic stimulation resulting from the other two treatments was not of sufficient intensity to bring about flower bud formation when the buds in the axils of the second and third compound leaves were still in condition to be influenced

in their development. A difference also occurred in the positions of the first flowers in the lots on 16-hour and continuous photoperiod. These results show that as the photoperiod is increased the length

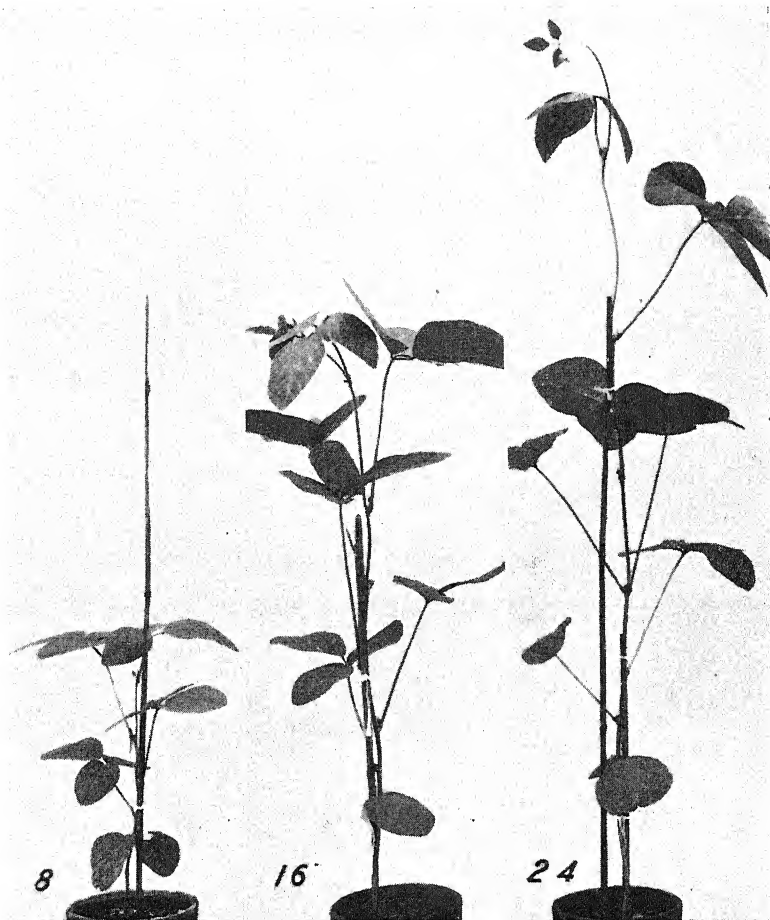


FIG. 8.—Batorawka soybeans grown on 8-, 16-, and 24-hour photoperiods respectively. Photographed May 3, 1939, 43 days after planting.

of time necessary for the initiation of flower primordia is also increased.

Somewhat similar responses have also been obtained with other varieties. The variety Ito San was grown on 14- and 18-hour photo-

periods from the time of emergence of the seedlings from the soil until the plants were harvested. The first flower primordia on the 14-hour lot were in the axil of the fourth compound leaf of most plants and in the axil of the third in the remaining ones. On the

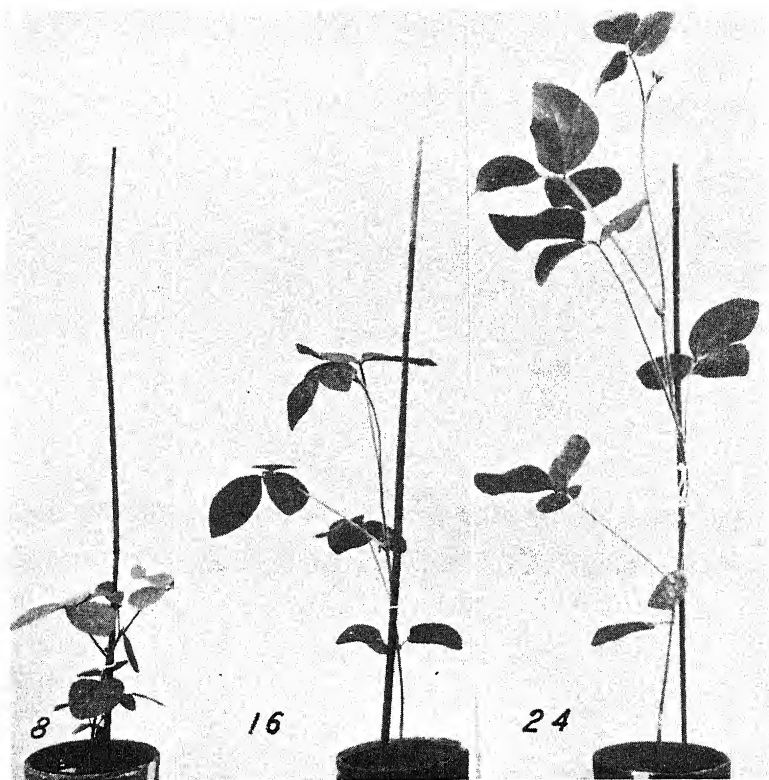


FIG. 9.—Mandarin soybeans grown on 8-, 16-, and 24-hour photoperiods respectively. Photographed May 3, 1939, 43 days after planting.

18-hour photoperiod the first flower primordia were in the axil of the twelfth leaf in most plants and in the axil of the thirteenth in the rest. The same type of response has also been observed in Biloxi. When grown on 8-hour photoperiod these plants initiate their first flower primordia in the axil of the second compound leaf, but when grown on photoperiods of 16 or more hours they initiate their first

flower primordia in the axil of the thirtieth leaf or at even higher positions. In all experiments in which Biloxi soybeans have been

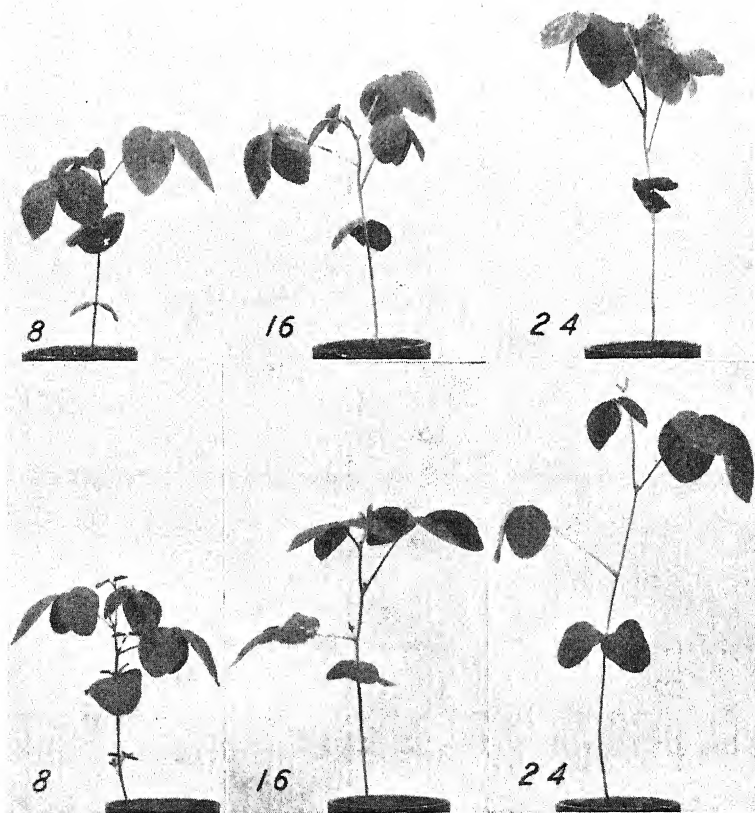


FIG. 10.—Biloxi and Agate soybeans grown on 8-, 16-, and 24-hour photoperiods respectively. Upper row: Biloxi; lower row: Agate. Photographed October 7, 1938, 31 days after planting.

grown for several months on photoperiods of 16 hours or more, the plants have ultimately initiated flower primordia.

The data concerning time and place of origin of first flower primordia in these different varieties may mean (1) that there is a slow

accumulation of a rather weak photoperiodic stimulus received from long photoperiods, or (2) that the plant becomes progressively more responsive to the same degree of stimulation as it grows older. Data supporting this last view have been published previously (2). No data have been obtained either for or against the other assumption.

#### CHEMICAL STUDIES OF BILOXI AND AGATE SOYBEANS

It has been shown that Biloxi soybeans remained vegetative and Agate soybeans promptly initiated flower primordia when grown on a 16-hour photoperiod. Since the two varieties made these different responses when grown under identical environmental conditions, they presented an opportunity to compare the metabolism of vegetative and reproductive plants of the same species that had received the same amount of total radiation.

For this experiment Biloxi and Agate seeds were planted in white quartz sand in four different control rooms. The planting was arranged so that alternate pots contained Biloxi seeds and the remaining pots Agate seeds. The pots were flushed daily with a four-salt nutrient solution which from previous experience gave good growth in the control rooms employed. Light was furnished by carbon arc lamps burning Sunshine carbons. The arc light was supplemented by Mazda light. The intensity of the carbon arc lights was approximately 1300 foot-candles in two rooms and 1800 foot-candles in the other two rooms. The intensity of the Mazda light used was 160 foot-candles in all rooms. The temperature was maintained at 70° F.  $\pm$  2° in all four rooms.

The plants were thinned to four per pot 10 days after planting. When the plants were 3 weeks old a composite sample of each variety was taken from all four rooms by collecting one plant from each pot. This procedure was repeated at the end of 4, 5, and 6 weeks, respectively. The plants used for the 2-week samples were grown in the same rooms under the same conditions, but since the plants were very small at 2 weeks, all of them were used for this sample. When harvested, the plants were cut at the cotyledonary node and the leaves and stems were separated. Twenty or more plants from every sample taken were examined morphologically.

No flower primordia were present on any Biloxi plants during the

entire experiment. In Agate there were no flower primordia at the end of the second week, but at the end of the third they were observed on eleven of twenty-four plants examined. At the end of the fourth and all subsequent weeks they were present on all Agate plants.

During the first 4 weeks the Agate plants differentiated new leaf primordia at the apex of the main stem more rapidly than did Biloxi. At the age of 2 weeks Biloxi had an average of 10.0 nodes per plant and Agate 10.3. At the end of 4 weeks Biloxi had 15.6 nodes and Agate 17.7. By the end of the fifth week terminal inflorescences had been formed in Agate plants. With the advent of these, differentiation of further nodes and internodes in the main stems ceased. The Biloxi plants continued to form new leaf primordia and at the end of 6 weeks had an average of 22.1 nodes per plant.

Throughout the entire period of the experiment the Biloxi plants were erect and sturdy with no evidence of branching. On the other hand the Agate plants had stems of smaller diameter, showed a tendency toward twining, and developed vigorous side branches before the end of the experiment.

The chemical methods, except those used for the determination of amide and nitrate nitrogen, have been reported previously (7). Amide nitrogen was determined in duplicate 50-ml. aliquots of the extract after they had been hydrolyzed with 3 ml. of concentrated sulphuric acid for 2.5 hours under reflex condensers. After hydrolysis the solutions were nearly neutralized with sodium hydroxide and the ammonia was determined by aeration as reported (7). The nitrate nitrogen method employed was the same as used previously (7), except that the determinations were made on the extracts after the amide nitrogen had been removed.

The results of the chemical analyses of Biloxi and Agate soybeans are shown in tables 4, 5, and 6. The percentage of dry weight in the leaves of Agate plants was greater than in the leaves of Biloxi throughout the experiment, and in the stems the same condition existed except at the end of the sixth week. Agate plants accumulated dry weight at a more rapid rate than Biloxi for the first 5 weeks, and this accumulation occurred in both leaves and stems. During the sixth week the Biloxi plants accumulated dry weight

much more rapidly than in previous weeks and at the end of the experiment exceeded the Agate plants in dry weight.

Results of the nitrogen analyses are shown in table 5. The percentage of total nitrogen in both varieties decreased with age in both leaves and stems. The leaves of the Biloxi plants had a slightly higher percentage of total nitrogen than the Agate leaves, throughout the experiment. In the stems the percentage of total nitrogen

TABLE 4  
PERCENTAGE MOISTURE AND DRY WEIGHT; AND FRESH AND DRY  
WEIGHT PER PLANT OF BILOXI AND AGATE  
SOYBEANS AT VARIOUS AGES

VARIETY	AGE (WEEKS)	PERCENTAGE MOISTURE		PERCENTAGE DRY WEIGHT		FRESH WEIGHT PER PLANT (GM.)			DRY WEIGHT PER PLANT (GM.)		
		LEAVES	STEMS	LEAVES	STEMS	LEAVES AND STEMS	LEAVES	STEMS	LEAVES AND STEMS	LEAVES	STEMS
Biloxi...	2	85.91	91.63	14.09	8.37	1.45	0.80	0.65	0.16	0.11	0.05
Agate...	2	85.49	91.16	14.51	8.84	1.42	0.66	0.76	0.16	0.09	0.07
Biloxi...	3	84.48	88.77	15.52	11.23	4.65	2.25	2.40	0.62	0.27	0.35
Agate...	3	83.65	88.49	16.35	11.51	4.48	2.38	2.10	0.63	0.24	0.39
Biloxi...	4	86.37	88.79	13.63	11.21	10.78	6.88	3.90	1.38	0.44	0.94
Agate...	4	85.02	87.43	14.98	12.57	11.21	6.13	5.08	1.56	0.64	0.92
Biloxi...	5	86.81	87.95	13.99	12.05	19.44	12.08	7.36	2.58	0.89	1.69
Agate...	5	84.90	85.73	15.10	14.27	22.06	12.17	9.89	3.54	1.41	2.13
Biloxi...	6	84.74	84.69	15.26	15.31	44.79	28.96	15.83	6.84	2.42	4.42
Agate...	6	84.29	85.17	15.71	14.83	42.17	22.65	19.52	6.45	2.89	3.56

in Biloxi was greater than that in Agate at ages of 3, 4, and 5 weeks. The greatest difference in the percentage of total nitrogen in both leaves and stems occurred when the plants were 4 weeks old. At this time all Agate plants were differentiating flower primordia while the Biloxi were strictly vegetative.

The percentage of soluble non-protein nitrogen in the leaves was approximately the same in both varieties at 2 and 6 weeks, but in the stems was greater in Agate than in Biloxi. When the plants were 3, 4, and 5 weeks old the Agate leaves and stems contained a lower

TABLE 5  
NITROGEN DISTRIBUTION IN LEAVES AND STEMS OF BILOXI AND AGATE SOYBEANS AT VARIOUS AGES  
RESULTS BASED ON PERCENTAGE DRY WEIGHT

VARIETY	AGE (WEEKS)	TOTAL NITROGEN		SOLUBLE NON-PROTEIN NITROGEN		AMINO NITROGEN		AMIDE NITROGEN		AMMONIA NITROGEN		NITRATE NITROGEN	
		LEAVES	STEMS	LEAVES	STEMS	LEAVES	STEMS	LEAVES	STEMS	LEAVES	STEMS	LEAVES	STEMS
Biloxi.....	2	6.884	5.675	1.675	3.835	0.468	1.565	0.071	0.621	0.014	0.024	0.184	0.370
Agate.....	2	6.671	5.848	1.626	4.423	0.461	1.832	0.062	0.679	0.014	0.023	0.331	0.520
Biloxi.....	3	5.573	3.357	1.263	1.897	0.580	0.757	0.045	0.276	0.019	0.009	.....	.....
Agate.....	3	5.284	3.214	1.144	1.755	0.459	0.669	0.030	0.226	0.012	0.009	.....	.....
Biloxi.....	4	5.473	3.060	1.467	1.766	0.528	0.642	0.044	0.134	0.007	0.009	0.029	0.036
Agate.....	4	4.766	2.434	1.055	1.241	0.347	0.549	0.020	0.095	0.007	0.000	0.020	0.040
Biloxi.....	5	4.896	2.733	1.365	1.610	0.479	0.722	0.064	0.216	0.014	0.008	0.129	0.191
Agate.....	5	4.424	2.004	0.962	1.044	0.459	0.511	0.046	0.112	0.020	0.014	0.060	0.105
Biloxi.....	6	4.463	2.012	1.219	1.052	0.446	0.575	0.131	0.248	0.020	0.013	0.111	0.104
Agate.....	6	4.347	2.158	1.216	1.268	0.405	0.519	0.108	0.256	0.013	0.007	0.121	0.195

percentage of soluble non-protein nitrogen than the Biloxi leaves and stems.

The percentage of amino nitrogen varied in both leaves and stems in the same manner as the soluble non-protein nitrogen. Amide nitrogen in the leaves of Biloxi plants was always higher than in Agate leaves, and was also higher in Biloxi stems at 3, 4, and 5 weeks than in Agate stems.

The percentage of ammonia nitrogen in the two varieties was not greatly different during the experimental period and the slight differences show no particular trend. The amount of nitrate nitrogen was much higher in the Agate than in the Biloxi plants at 2 weeks. In later samples the differences were not great and showed no particular trend.

In general the nitrogen content of the two varieties was not greatly different. Such differences as occurred were greatest when the plants were 3 and 4 weeks old and it was at this time that floral initiation started to occur in the Agate variety.

Results of the carbohydrate analyses are shown in table 6. The percentage of reducing sugars in the leaves of the Agate plants was greater than in Biloxi at 3, 4, and 5 weeks. The percentage of reducing sugar in the stems was higher in Agate than in Biloxi in all except the 6-weeks sample. Differences in relative amount of total sugars in the two varieties were similar to differences in reducing sugars. The percentage of sucrose in the leaves did not differ greatly in the two varieties until the fourth week. At this time the Agate leaves contained much less sucrose than the Biloxi, but the stems contained more.

The percentage of starch in the Agate leaves was much greater than in the Biloxi leaves until the sixth week. The maximum amount was found in Agate at 3 weeks and in Biloxi at 6 weeks. In the stems the differences in percentage of starch in the two varieties were not so great and a maximum percentage occurred in both at 3 weeks.

The total carbohydrate content of Agate reached a maximum when the plants were 3 weeks old. In older plants there was a progressive decrease. On the other hand, in Biloxi the highest total carbohydrate content during the experiment occurred when the plants were 6 weeks old.

Since it is not known whether this difference in the rate of carbohydrate accumulation exists in all varieties that initiate flower primordia on any photoperiod, no general conclusions can be drawn. Nevertheless maximum total carbohydrates occurred in the leaves of Agate plants at the time floral initiation was beginning. While

TABLE 6  
CARBOHYDRATE CONTENT OF LEAVES AND STEMS OF BILOXI AND  
AGATE SOYBEANS AT VARIOUS AGES. RESULTS BASED  
ON PERCENTAGE DRY WEIGHT

VARIETY	AGE (WEEKS)	REDUCING SUGARS		TOTAL SUGARS		SUCROSE		STARCH	
		LEAVES	STEMS	LEAVES	STEMS	LEAVES	STEMS	LEAVES	STEMS
Biloxi.....	2	1.228	1.649	1.937	1.995	0.575	0.179	2.051	1.242
Agate.....	2	1.096	2.012	1.833	2.489	0.606	0.283	4.225	1.663
Biloxi.....	3	2.133	3.277	3.106	4.265	0.792	0.748	4.542	2.315
Agate.....	3	2.538	4.327	3.553	5.334	0.819	0.756	9.229	2.163
Biloxi.....	4	2.825	3.336	3.661	3.871	0.631	0.312	2.348	1.061
Agate.....	4	4.139	5.418	4.546	6.253	0.193	0.541	6.242	1.496
Biloxi.....	5	2.623	2.738	3.431	3.618	0.615	0.672	2.216	1.021
Agate.....	5	4.172	4.520	4.828	5.508	0.437	0.722	4.848	1.380
Biloxi.....	6	4.430	3.494	5.747	4.585	1.048	0.875	5.138	1.986
Agate.....	6	3.450	3.001	4.481	4.154	0.815	0.944	4.239	1.200

this condition is not thought to be the cause of floral induction, it may be correlated with the metabolism in some manner to promote the reaction or reactions causing induction.

### Discussion

The results indicate that early varieties of soybeans are unsuited to many studies of photoperiodic induction because their initiation of flower primordia is not photoperiodically limited. Although floral initiation occurs on all photoperiods, the further development of the flower primordia into flowers and fruits is delayed or completely inhibited by long photoperiods. It is therefore unsafe to depend upon macroscopically visible flower buds as an indication of the effectiveness of various treatments designed to influence floral initia-

tion. If such criteria alone are used, the effects of photoperiodic treatment upon induction and upon subsequent development cannot be separated. It is important that the absence of flower primordia on control plants be assured through microscopic examination of the growing points of an adequate number of such plants. It is also more satisfactory to use varieties such as Biloxi that do not initiate flower primordia on long photoperiods until they are many months old.

In earlier work with the Biloxi soybean (1) it has been demonstrated that single dark periods varying in length from 2 to 10 days are ineffective in bringing about photoperiodic induction. Not only must there be short light periods but the intensity of these must be above a minimum level of approximately 100 foot-candles if induction is to occur. These responses indicate that light periods have an important relation to photoperiodic reaction. It has also been found that variations in temperature of the dark periods during induction produce marked differences in the effectiveness of the induction treatment. From this it seems that important photoperiodic reactions may also occur during the dark periods.

The results of the present studies show that in many varieties of soybeans darkness is not a necessary condition for floral initiation. It has been shown, however, that in a variety such as Agate, in which floral initiation is least limited by continuous light, initiation occurs much more promptly if the plants are subjected to periods of darkness. The Biloxi variety, which during its early weeks of growth does not initiate flower buds unless it receives daily dark periods of approximately 10 hours or more, finally reaches a condition after many weeks in which it is capable of forming flower primordia on considerably shorter dark periods. It is not improbable that flower primordia might be formed on the old plants of this variety grown on continuous light. The two varieties do not appear to differ in the fundamental character of the reactions that control floral initiation. They differ markedly, however, in the relative ages at which they make a similar response to long photoperiod. During the first 6 weeks, over which period chemical data are available,

they also differ in chemical composition. Agate reached a maximum in total carbohydrates at 3 weeks while the trend of the data for Biloxi seemed to indicate that a maximum had not yet been reached at the close of the experiment.

### Summary

1. A study of floral differentiation has been made upon thirteen varieties of soybeans grown on nine different photoperiods, including continuous light. The varieties Agate, Mandell, Hudson Manchú, Minsoy, Mc.Crostie's Mandarin, Wisconsin Early Black, Batorawka, and Mandarin initiated flower primordia on all photoperiodic treatments employed. These varieties normally mature seed in 105 days or less under field conditions. No plants of the varieties Biloxi, Avoyelles, Ootootan, and Peking, and few of Tokyo, initiated flower primordia on photoperiods longer than 16 hours. These varieties require 125-175 days to mature seed in the field.

2. The initiation of flower primordia in many varieties under continuous light shows that darkness is not a necessary condition for floral initiation in them.

3. In Agate, one of the varieties that initiates flower buds most readily on continuous light, the reactions causing induction are accelerated if the plants are subjected to daily periods of darkness. Although these reactions take place in continuous light they proceed more rapidly if there is a daily alternation of light and dark.

4. Chemical analyses of the varieties Biloxi and Agate have been made at the ages of 2, 3, 4, 5, and 6 weeks. In general Agate contained less total and less soluble forms of nitrogen and more carbohydrates than Biloxi. The starch content of Agate reached a maximum at 3 weeks and declined progressively with age, while in Biloxi the trend of the data indicated that a maximum had not been reached during the experiment. The maximum carbohydrate content in Agate occurred at the time floral initiation was beginning.

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# EFFECTS OF TEMPERATURE, CALCIUM AND ARSENOUS ACID ON SEEDLINGS OF *POA PRATENSIS*

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 506

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(WITH THREE FIGURES)

## Introduction

The natural range of Kentucky bluegrass over the northern half of the United States and southern Canada indicates that it is adapted to cool conditions. In addition, it has been observed (12) that bluegrass grows slowly in hot weather and, when well established, most vigorously (6) at temperatures ranging between 15° and 25° C. Also it is known that bluegrass will flourish and grow well on calcareous soils, either because of their lime content or because of other associated nutritive conditions.

During recent years there have been reports that certain chemicals, other than those usually considered as nutrients, will stimulate growth. Thus arsenic salts have been found in some cases to effect a distinct increase in growth of well established bluegrass. These observations were made, as has been most of the work, on mature plants.

Some preliminary work, however, has indicated that Kentucky bluegrass, once it has become established, will grow well under conditions which are unfavorable for the germination of its seeds and for early growth. In the experiments reported here attention is directed primarily to the effect of temperature, calcium content of soil, and arsenic on germination and on growth of seedlings.

## Material and methods

Kentucky bluegrass seeds of high purity were planted in washed white quartz sand contained in 2-gallon glazed crocks. Three gm. of seeds were used in each crock, except in the final calcium carbonate series in which 2 gm. were used. The seeds were evenly distributed,

then lightly covered with sand and watered daily with distilled water until germination began. Each pot then received bi-weekly applications of a modified Shive's solution (22) to which had been added 0.5 ppm  $\text{ZnCl}_2$ ,  $\text{H}_3\text{BO}_3$ , and  $\text{MnCl}_2$ , 0.125 ppm of  $\text{CuCl}$ , and traces of ferric citrate.

While making trial runs it was found that fine quartz sand was unsuitable for winter work, perhaps because damping-off fungi thrived under the prevailing warm, moist, cloudy conditions. The most successful practice was to use coarse quartz sand and to spray the seeds at the time of planting and at frequent intervals thereafter with a dilute Semesan solution.

At the termination of the experiments dealing with calcium carbonate fresh and dry weights of the grass were taken. Ninety days after the experiment was started half the pots in each series were sampled to determine the relative number of plants which survived; plants from 1 cm. squares along two diameters of each pot were counted.

### Investigation

#### A. EXPERIMENTS WITH CALCIUM CARBONATE

A series of preliminary experiments were carried out in an attempt to study the effect of calcium carbonate on germination, but the results were so varied that the details are not given here.

In the final lot of the calcium carbonate series, started March 20, 1938, thirty-three pots were filled with sand which had approximately  $\frac{1}{2}$  pound of calcium carbonate per pot mixed with it. The solution which dripped from the pots after watering had a reaction of pH 8.0. Although the calcium carbonate-sand mixture seemingly produced a well buffered medium, the pots were watered at intervals with water heavily charged with calcium carbonate to avoid any chance of a decrease in pH during the course of the experiment. Twenty-eight pots were set up comparably except that no calcium carbonate was added. The reaction of the leachate of the latter pots, as shown by colorimetric tests, was fairly constant at pH 5.6.

After studies were made concerning the effect of high calcium carbonate content of the sand on germination and early growth of grass; that is, some 35 days after germination, the plants of twenty-

one plus- $\text{CaCO}_3$  and of eight minus- $\text{CaCO}_3$  pots were harvested. During these early stages of growth the temperature of the sand had not been controlled, but the remaining pots were placed in tanks which kept soil temperatures constant to within  $1^\circ \text{C}$ . Half were maintained at a soil temperature of  $15^\circ \text{C}$ .; the other half at  $25^\circ \text{C}$ . Fifty-five days after making the temperature adjustments photographs were taken and harvests made.

### B. EXPERIMENTS WITH ARSENOUS ACID

During the months of January to March a series of experiments designed to show the effects of arsenous acid on germination and on seedling growth were set up in a greenhouse maintained at a temperature of approximately  $15^\circ \text{C}$ . In the first experiment five pots constituted a unit; each unit was sprayed with a liter of one of the following solutions: 0.01, 0.025, 0.05, 0.1 per cent, distilled water. The seeds were then sprinkled lightly with fine sand and moistened with distilled water.

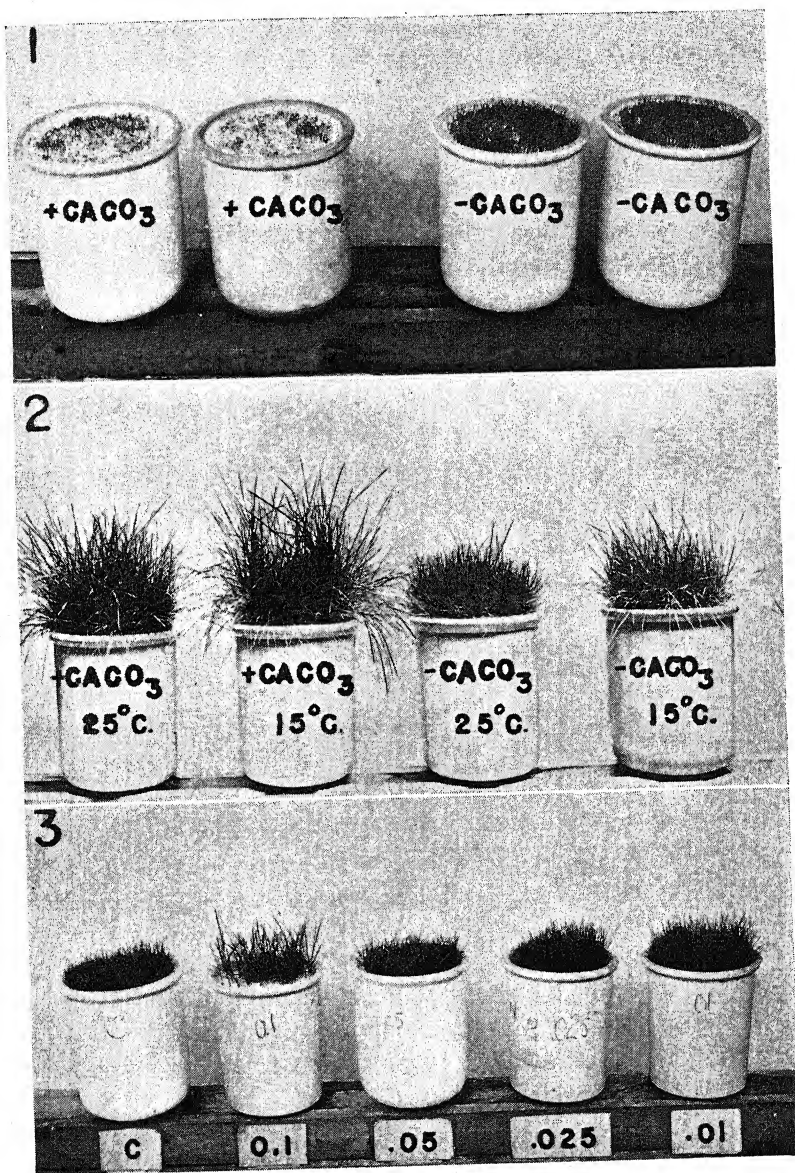
In a second experiment two pots composed a unit. The grass was grown until  $1\frac{1}{2}$  months old and the same treatment given. In a third experiment the grass was  $3\frac{1}{2}$  months old before treatment. All three experiments were continued for 3 months following the initial treatment.

## Results

### A. EFFECT OF CALCIUM CARBONATE ON GERMINATION

In the initial trials of this experiment it was very difficult to get an even stand with the various treatments. In the final one, germination began 6 days after planting (March 26) in the pots lacking calcium carbonate. There were no signs of germination in the other pots. This same condition had been observed, in a less pronounced form, in a previous experiment.

Germination was delayed approximately a week in the pots containing calcium carbonate (fig. 1). After germinating, the grass would grow for only a few days and then some would wither, leaving isolated clumps. If moist chambers were placed over individual pots further germination ensued and fairly good growth followed. Even with this treatment, however, some grass died. It is not likely that



FIGS. 1-3.—Fig. 1, effect of calcium carbonate content of soil on germination of bluegrass seedlings (13 days after planting). Fig. 2, effect of calcium carbonate content of soil and temperature on growth (90 days after planting). Fig. 3, effect of arsenous acid on germination and early growth. First pot on left received no acid; pots from 0.1 to 0.01 received the indicated concentrations at the rate of 1 liter per five pots.

death can be attributed to damping-off fungi because that disease produces different symptoms.

The retardation in germination of seeds observed in the plus calcium carbonate series is in agreement with the work of RUDOLFS (20) and BORTHWICK (3). The former found that calcium nitrate had a detrimental effect upon germination and root length of a number of plants; the latter, using single and two-salt solutions, found that germination of *Hypericum perforatum* was decreased almost proportionally as the amount of calcium was increased. BORTHWICK's data indicated that pH did not affect germination to any extent but that calcium did, perhaps by changing in some way the permeability of the seed coat to water.

Shortly after germination in the calcium carbonate series the leaves appeared to be vigorous but the root systems of the seedlings were poorly developed. This may be contrasted with the grass grown without calcium carbonate, which germinated in about 6 days, produced leaves, and developed root systems rapidly.

Slow root development of the germinating seedlings in the calcium carbonate series may possibly have been caused by the low availability of phosphorus at the high pH. Phosphorus is considered necessary for development of roots, and some work with wheat seedlings (7) has indicated that its uptake is greatest in the early stages of development. It is possible that in this experiment it was a limiting factor in the early stages. Later, absorption of phosphorus was sufficient for more extensive growth.

Thirty-five days after planting, twenty-one pots treated with calcium carbonate and eight not so treated were harvested. Average lengths of tops and roots and dry weights were taken.

From table 1 it is seen that, on the basis of weight of both tops and roots, the stand of grass grown in pots at pH 8.0 was not nearly so great as that grown at pH 5.6. Nevertheless, average length of roots in the series high in calcium carbonate was almost twice as great as in the minus calcium carbonate series. Also the tops were about 1.5 cm. longer in the former series than in the latter.

The root systems of the seedlings grown at pH 8.0 were characteristically long with few or no secondary roots. Where secondary roots were present, they were short except near the growing point of

the primary root. In contrast with this, seedlings grown at pH 5.6 had shorter primary roots and a better development of secondary roots in the more mature region. Those roots grown in the presence of calcium carbonate had become fibrous in the upper portion. Most of the root was thick and white, however, indicating that it was young. The roots grown in the absence of calcium carbonate were thin and suberized almost to the tip.

Once growth had started in the pots containing calcium carbonate the plants grew more rapidly, and as uniformly (as to tops and roots) as those not having calcium carbonate. CLEVINGER (4) found

TABLE 1  
EFFECT OF PRESENCE OF  $\text{CaCO}_3$  ON GROWTH OF BLUEGRASS SEEDLINGS  
35 DAYS AFTER PLANTING

TREATMENT	NO. POTS	PORTION OF PLANT	AVERAGE LENGTH (CM.)	DRY WEIGHT (GM.)	AVERAGE DRY WEIGHT PER POT (GM.)	TOP/ROOT RATIO
High $\text{CaCO}_3$ (pH 8.0)	21	{Tops Roots}	6.6 8.38	24.2 46.5	1.15 2.21	0.52
No $\text{CaCO}_3$ (pH 5.6)	8	{Tops Roots}	5.02 4.8	16.8 48.4	2.1 6.05	0.347

with oats and legumes that liming was correlated with more vigorous development. Calcium carbonate, perhaps by stimulating the natural metabolic and growth processes, is associated with a rise in the production of acids; this of course would decrease the internal pH. Such a decrease was found by BONNER in his work on *Avena coleoptile* (2) to be associated with an increase in growth substances. If this is true, more growth might be expected when calcium carbonate is present.

#### B. COMBINED EFFECTS OF TEMPERATURE AND CALCIUM CARBONATE CONTENT OF SUBSTRATE ON GROWTH

After the harvest, soil temperature experiments were started using the remaining pots. At the end of 55 days of growth photographs were taken and harvests made.

In general, top growth was greatest when the soil temperature

was 15° C., and the plants tended to be longer and to have more succulent blades and darker green leaves than grass grown at 25° C. (fig. 2). However, percentages of water in the leaves of the plants of the two temperature series show very little difference. This is at variance with the work of DARROW (6), who found in mature bluegrass that low temperature plants contained the most water per unit weight.

The grass grown in sand having a high calcium carbonate content had longer and darker green leaves than that grown on a substrate lacking lime. Furthermore, the dry matter produced was almost 33 per cent greater (table 2). The calcium carbonate series produced (although there were only about one-third the number of plants per pot) a total dry weight of tops approximately one-third greater than did those pots lacking calcium carbonate. Thus it becomes apparent that individual seedlings increase many more times in dry weight when calcium carbonate is present than when absent (table 3).

When the effects of temperature and calcium carbonate are considered, it is found that at 15° C. the presence of calcium carbonate is associated with an increase of 77.3 per cent in dry weight, and at 25° C. with 135.6 per cent, calculations being based on the dry weight of 100 seedlings.

The densest, most finely branched, and longest root systems were produced in the substrate having a high calcium carbonate content and grown at 15° C. On the basis of dry weight (table 2) the second best root system was produced in the absence of calcium carbonate and at a temperature of 15° C. These latter roots made up a fine meshwork, but they were not so near the surface as those just described; however, they were approximately as long. The roots of seedlings grown in the presence of calcium carbonate at 25° C. were somewhat similar to those grown without lime at 15° C. In general the roots were more slender, not so long, and the surface mat formation only fair. Least root growth was made by grass grown at 25° C. and lacking calcium carbonate in the substrate. Lateral growth just beneath the surface was very slight, and the roots had not penetrated to the bottom of the pots.

Table 2 indicates that low temperature is of much more importance in resulting development of abundant root growth than is

the calcium carbonate content of the substrate. In the temperature experiment it was found that root weight was 76.6 per cent greater at 15° than at 25° C. when calcium carbonate was present. Similarly growth was 91 per cent greater at 15° than at 25° C. when calcium carbonate was absent. It was found, however, that root growth was about 26.85 per cent greater when calcium carbonate was present in the soil at 15° C. than when it was not; and that it was 31.7 per cent greater when calcium carbonate was present in the soil at 25° C. than when it was absent.

TABLE 2  
RELATIVE EFFECTS OF  $\text{CaCO}_3$  AND TEMPERATURE ON DEVELOPMENT  
OF KENTUCKY BLUEGRASS SEEDLINGS

SOIL TREATMENT	SOIL TEM- PERA- TURE (°C.)	No. POTS	WEIGHT ROOTS (GM.)		AVERAGE DRY WEIGHT ROOTS PER POT (GM.)	WEIGHT TOPS (GM.)		AVERAGE DRY WEIGHT TOPS PER POT (GM.)	TOP/ROOT RATIO*
			GREEN	DRY		GREEN	DRY		
High $\text{CaCO}_3$ (pH 8.0)...	15	5	168.6	92.2	18.44	215.2	76.6	15.32	0.83
	25	5	114.0	51.6	10.32	206.5	76.3	15.26	1.48
No $\text{CaCO}_3$ (pH 5.6)...	15	9	243.7	121.3	13.48	300.7	108.1	12.01	0.89
	25	9	136.5	63.5	7.05	256.9	97.7	10.85	1.53

\* Calculated on basis of average dry weights per pot.

In the experiment considered here, plants having much greater green and dry weights, the greenest color, longest leaves, and a low top/root ratio were obtained in cultures grown at 15° C. Dry weights obtained by DARROW indicated that the optimum temperature for growth of mature bluegrass ranges between 15° and 25° C., while HARRISON'S (13) data from clippings indicated that the optimum temperature for top growth was 15° C.

The top/root ratios (table 2) were predominantly influenced by temperature. Those plants grown at 25° had much poorer root development than those grown at 15° C. This difference cannot be attributed to a difference in degree of competition, because there were relatively equal numbers of plants per pot in the temperature series. On the basis of these figures, calcium carbonate content of the substrate played a negligible role in altering top/root ratios. On the

other hand, dry weight figures are correlated with a marked increase in general metabolism when calcium carbonate is present.

Rhizome development, which was just beginning at the time of the harvest, was most noticeable in the pots with open and sparse growth.

The general conclusions from this experiment are that calcium in large quantities is detrimental to the germination and growth of young seedlings. Thus it would appear that heavy liming with calcium compounds before seeding would be inadvisable. Once seed-

TABLE 3  
RELATIVE NUMBERS AND WEIGHTS OF SEEDLINGS AS  
CORRELATED WITH TEMPERATURE

SOIL TREATMENT	SOIL TEMPERA- TURE (° C.)	NO. OF 1 CM. <sup>2</sup> SAMPLES TAKEN*	NO. PLANTS PER SQ. CM.	APPROXI- MATE NO. SEEDLINGS PER POT	DRY WEIGHT OF 100 SEEDLINGS (GM.)†
High CaCO <sub>3</sub> (pH 8.0)....	{15	56	5.4	1696.5	9.4
	{25	64	5.5	1721.6	7.4
No CaCO <sub>3</sub> (pH 5.6).....	{15	58	13.6	4272.58	5.3
	{25	63	16.3	5120.81	3.14

\* Samples taken at random along diameters of pots at right angles to one another.

† Calculated.

lings are established, however, the application of lime would tend to stimulate growth greatly. With application of calcium carbonate there is an increase in fine roots near the surface, tending to limit the coming in of species which would have to compete for water and nutrients. In soil containing calcium carbonate, roots grow longer, which makes the grass less likely to die as a result of the drying out of upper soil layers.

Planting of seeds during periods of decreasing temperatures, as in the early autumn, should prove most favorable for promoting establishment, for these experiments show that superior root development is attained at low temperatures. Since an extensive root system is necessary for survival through dry periods of the summer, roots would have a better opportunity for such development if seeds were sown in the autumn rather than the spring.

## C. EXPERIMENTS WITH ARSENOUS ACID

Sixteen days after planting and spraying the seeds there was sporadic germination of those sprayed with distilled water, and some germination of those sprayed with the lowest concentration (0.01 per cent). Two days later germination began in pots sprayed with 0.025 per cent arsenous acid. Thereafter, germination occurred in decreasing amount in the pots treated with 0.05 and 0.1 per cent acid. By the end of 27 days it was apparent that the grass treated with 0.01 per cent arsenic was germinating much more completely than the controls, while that treated with 0.025 per cent was germinating about as well as the controls. There was definite retardation in the pots treated with 0.05 per cent arsenous acid, and very marked toxicity to seeds was shown in pots treated with 0.1 per cent, the highest concentration (fig. 3). The stimulating effects continued to be apparent after 2 months in the case of the grass treated with the 0.025 and 0.01 per cent.

The slowness of germination of all the grass in the arsenic experiments may be partially attributed to the low temperature of the greenhouse. An essentially constant temperature of 18° C. was maintained throughout the day. Low constant temperatures are not most conducive to germination of Kentucky bluegrass, as was shown by HARRINGTON (11); VANHA (24) found that temperature alternations between 20° and 32° C. are best for rapid germination.

In the experiments with the 1½ and 3½ months-old grass it was observed 5 days after spraying that there was injury from the 0.1 per cent solution, the leaves appearing somewhat dried. A week after spraying, injurious effects became apparent with the 0.025, 0.05, and 0.1 per cent solutions, the most severe being brought about by the highest concentration. In general the oldest grass was slowest to respond. Within 2 weeks it was apparent that the grass treated with 0.1 per cent arsenous acid would die, while in the remaining treatments, although there was injury, the severity decreased with decreasing concentration.

Slightly over a month after treatment with the various arsenous acid sprays, the older grass treated with 0.05 and 0.1 per cent solutions was almost completely killed. Thus an upper tolerance of *Poa*

*pratensis* for arsenous acid, when used as a spray, was found to be a 0.05 per cent solution. Some seeds germinated which had previously remained dormant. Practically all the seeds germinating in the pots treated with 0.1 per cent arsenous acid were weeds present in the seed sample, but no weed seedlings were observed in the pots sprayed with the lower concentrations. Apparently the higher concentrations stimulated germination of weed seeds. Another effect noted was that the grass sprayed with the lower concentrations was more drought resistant than the controls.

After a few weeks the older grass, which had not been injured severely, began to recover from the initial effects of arsenic "burning." The grass treated with 0.01 per cent arsenous acid, however, showed no adverse effects from spraying; there seemed to be a slight benefit.

The purpose of the arsenous acid experiments was to determine whether or not arsenic had a direct stimulatory effect on Kentucky bluegrass. The literature is filled with conflicting statements as to the effects of arsenic on different plants. Some investigators (1, 8, 14, 23) have maintained that arsenic, when present in small quantities, acts as a stimulating agent to growth, while others (5, 17, 18, 19) have found no stimulating effects. All agree that when arsenic is present in large quantities it is toxic. Very little work has been done to indicate whether the observed stimulations were direct or indirect. Some work by GREAVES (10) indicated that there was stimulation of soil bacteria in that there was a comparatively greater production of available nitrogenous compounds in the soil caused by the addition of arsenic. The stimulation to growth he observed may also have been caused by the inhibition of injurious bacteria and fungi. Also, according to GREAVES, arsenic can in some way liberate phosphorus from its insoluble compounds, thus exerting another indirect action.

It has long been known that different plants are differentially subject to arsenic poisoning. For example, legumes are relatively susceptible while rye is rather tolerant (21). HUTCHESON and WOLF (15) reported the use of large quantities of sodium arsenite in the control of hawkweed without injuring Kentucky bluegrass. Arsenic compounds have been found (9, 16) to be excellent as selective

eradicants of clover, chickweed, speedwell, and a few other low-growing weeds, while not injuring bluegrass. Toxicity is apparently most pronounced in shallow rooted species (9). In the experiments reported here seeds appear to be differentially affected by arsenous acid, a 0.1 per cent solution killing nearly all bluegrass seeds while seeds of weed grass, which were present as impurities, were apparently stimulated to germinate and grow.

### Summary

1. Germination of seeds in the pots having a high calcium carbonate content (pH 8.0) began about a week after that in the pots having no calcium carbonate (pH 5.6).
2. Thirty-five days after planting, the secondary roots formed at the time of germination continued to live in the minus calcium carbonate series, while there were few or none when calcium carbonate was present.
3. The average length of roots was almost twice as great when calcium carbonate was present as when absent.
4. Ninety days after planting, top growth of grass grown on a substrate having a high calcium carbonate content was, on the basis of dry weight, approximately 33 per cent greater than when calcium carbonate was absent.
5. When the effects of temperature and calcium carbonate are considered, it is found that at 15° C. the presence of calcium carbonate is associated with an increase of 77.3 per cent in dry weight, and at 25° C. with 135.6 per cent, calculations being based on the dry weight of 100 seedlings.
6. Data indicate that calcium carbonate content of the soil is not of as great importance in producing extensive root growth in seedlings as is temperature. During the period of this experiment root growth was definitely greater at 15° than at 25° C.
7. The presence of calcium carbonate in the substrate was correlated with an evenly increased metabolic activity and growth regardless of temperature. This is especially reflected in the top/root ratios.
8. Under conditions of high temperature (22°-23° C.) liming of

the soil shortly after germination has taken place may be most favorable for the establishment of a good stand.

9. Time of germination was not delayed by the arsenic when applied as a 0.01 per cent solution, but with increasing concentration there was increasing retardation. With the lowest concentrations germination and growth were greater than in the control pots.

10. In Kentucky bluegrass an upper tolerance was found for arsenous acid (0.05 per cent), a single application of 0.1 per cent solution being sufficient to kill seeds and seedlings.

11. Harmful effects of arsenous acid were progressively less with decreasing concentration from 0.1 to 0.01 per cent.

12. Bluegrass treated with a 0.01 per cent arsenous acid solution showed greater germination than did untreated seeds. A thicker and taller stand was produced in a shorter time and this advance was maintained throughout the experiment.

13. Considerably higher concentrations of arsenous acid are required to kill the growing point of the stem than to kill the leaves.

14. Arsenous acid has a greater toxicity to Kentucky bluegrass seeds than to the seeds of some other grasses.

The writer wishes to express his appreciation of the helpful suggestions given by Dr. K. C. HAMNER during the course of these experiments.

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A MORPHOLOGICAL STUDY OF AMPLISIPHONIA  
A NEW MEMBER OF THE RHODOMELACEAE

GEORGE J. HOLLENBERG

(WITH THIRTEEN FIGURES)

A number of times during the last several years the writer has collected a striking marine red alga, for which the following diagnosis is given.

*Amplisiphonia* gen. nov.

Frond membranaceous, prostrate, horizontally expanded and irregularly lobed, attached to rocks by means of numerous unicellular rhizoids; growth marginal as a result of the activity of a row of apical cells; thallus unistratose at the margin and composed of radiating dichotomous rows of cells cut off from the apical cells, soon polysiphonous as a result of the cutting off of three upper and two lower pericentral cells from each central cell; tetrasporangia arising in modified lobes of the frond which soon become erect and are often much ruffled and bear the tetrasporangia in radiating rows; tetrasporangia arising from a special pericentral cell, more or less cruciately divided; three pericentral cells are cut off above and three below each central cell in fruiting portions; cystocarps and antheridia unknown.

Frons membranacea, prostrata, horizontaliter expansa et irregulariter lobata, ad saxa per rhizoidea numerosa unicellularia fixa; per cellulas apicales marginales crescens; margine thalli unistrato, ex seriebus radiatis et bifidis cellularum constructo; siphonibus quinque (tribus superioribus et duobus inferioribus), a quaque cellula centrale mox praecisis; tetrasporangiis in lobis modificatis prostrato-erectis frondis immersis, in seriebus radiatibus instructis, ex cellulis pericentralibus orientibus, subcruciatim divis; in partibus fructiferis tribus cellulis inferioribus et tribus superioribus a quaque cellula centrale praecisis; antheridiis et cystocarpiis ignotis.

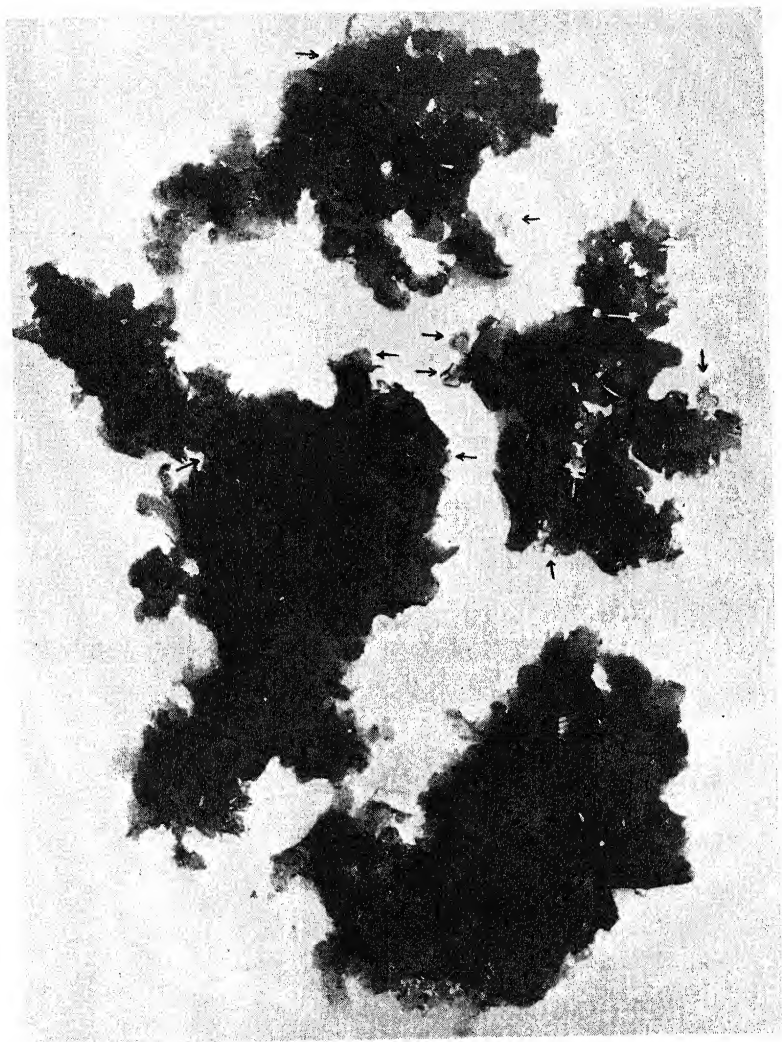


FIG. 1.—Portions of type specimen of *Amplisiphonia pacifica* gen. et sp. nov. Slightly enlarged. Arrows indicate fruiting lobes.

*Amplisiphonia pacifica* sp. nov.

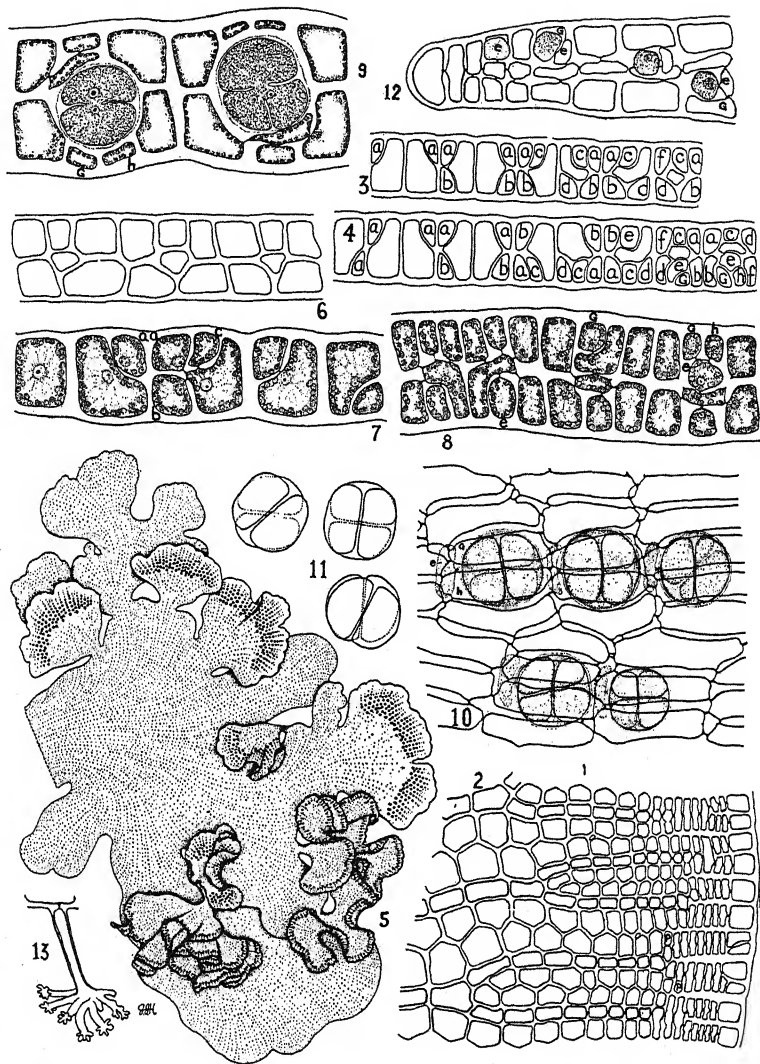
Frond prostrate, forming deeply and irregularly lobed, rosette-like expansions 10–15 cm. or up to 5 dm. across and 140  $\mu$  thick; medium or dull red in color; fruiting lobes marginal, narrowly attached, at first obovate-cuneate, prostrate and plane, later erect, ample and ruffled at the margin, up to 5 mm. high and 6 mm. or more broad.

Frons prostrata, expansa et irregulariter profuseque lobata, usque 10–15 cm. aut ad 5 dm. lata et 140  $\mu$  crassa; color nonnihil subfuscus ruber; lobis fructiferis ab margine frondis ortis, primum obovato-cuneatis et prostratis et planis, mox erectis et amplis et plicatis, ad 5 mm. altis et 6 mm. latis.

This interesting member of the Rhodomelaceae, hitherto undescribed, is frequently encountered on rocky shores along the coast of southern California. It is usually closely attached to crustaceous corallines in the lower littoral zone. It has been found clambering over the hapteres of *Eisenia*. It has been collected at all seasons of the year. It spreads in a vegetative manner and often covers a considerable area on the substrate. No. 2515, in the herbarium of the writer, has been selected as the type specimen. It is believed to represent a single plant which had spread over an irregular area 5 dm. or more in diameter. This opinion is based on the fact that, although the plants are infrequently found in fruit, in this case an abundance of fruiting lobes were present throughout the entire area covered by the plant. Only tetrasporic plants have been found so far. Most of the plants collected are sterile. Figure 1 represents portions of the type specimen collected at Corona del Mar, California, February 2, 1939. *Amplisiphonia* has been collected as far north as the Monterey region.

### Methods

Both vegetative and reproductive portions of *Amplisiphonia* were fixed in a solution composed of 1 gm. chromic acid and 1 cc. of glacial acetic acid per 100 cc. of sea water. N-butyl alcohol was employed in dehydrating and imbedding, and serial paraffin sections 6  $\mu$  thick were made in the usual manner and stained with Heidenhain's iron-alum haematoxylin.



FIGS. 2-13.—All drawings made with Abbé camera lucida except sketch for fig. 5 and diagrams for figs. 3 and 4. Fig. 2, portion of margin of frond of *Amphisiiphonia pacifica* showing apical cells and dichotomous cell rows and veins. Position of latter indicated by heavier lines around cells immediately above central ones;  $\times 325$ . Fig. 3, diagrammatic representation of vertical section of frond near and parallel with margin, showing manner of origin of pericentral cells in vegetative portions. Fig. 4, same of fruiting lobe near and parallel with margin, showing order of origin of pericentral cells and manner of origin of supporting and cover cells. Fig. 5, part of plant of *A. pacifica* showing fruiting lobes;  $\times 2.5$ . Fig. 6, transection through mature portion of frond;  $\times 325$ . Fig. 7, vertical section of tetrasporic lobe of frond near and parallel with margin, showing origin of first pericentral cells;  $\times 700$ . Fig. 8, same of fruiting lobe near and parallel with margin, showing final stages in formation of pericentral cells, supporting cells (e), and cover cells (g, h);  $\times 700$ . Fig. 9, same of older fruiting portion of frond perpendicular to length of cell rows, showing tetrasporangia forming tetraspores;  $\times 325$ . Fig. 10, surface view of fruiting region showing tetrasporangia, supporting and cover cells;  $\times 162$ . Fig. 11, arrangement of tetraspores in tetrasporangia dissected from thallus;  $\times 162$ . Fig. 12, longitudinal section of cell row at margin of frond, showing apical cell and manner of origin of tetrasporangia;  $\times 700$ . Fig. 13, mature rhizoid showing

## Description

### VEGETATIVE STRUCTURE

Growth in *Amplisiphonia* is limited to the margin of the frond and is the result of the activity of a row of relatively large marginal cells which cut off new cells by transverse divisions (fig. 2). These marginal cells measure up to  $12\ \mu$  long and  $9\ \mu$  wide in actively growing regions. Frequent oblique divisions of the apical cells give rise to new cell rows, and as a result the lobes of the thallus are usually round in outline. New lobes arise from the margins of old ones as a result of a local acceleration of the activity of the marginal row of cells. Radial growth of the irregularly overlapping lobes results in a rosette-like thallus in the case of older plants. The plants are perennial, and central portions of older plants are commonly composed of a number of layers of the multi-lobed thallus, each successive layer growing over and attaching to the next lower layer. The apical cells are increasingly more active toward the distal portion of each lobe, but in any limited portion of the margin the apical cells are quite uniformly active, so that the margins of the rounded lobes usually remain relatively smooth in outline (fig. 2), although they are sometimes very irregular.

At the margin the thallus is unistratose, but it soon becomes 2-3 cells thick and polysiphonous in structure. The cells resulting from the transverse divisions of the marginal cells give rise to a row of central cells from each of which five pericentral cells are cut off in vegetative portions. In older parts of the thallus the rows of central cells appear as microscopic forking veins. In figure 2 these veins are suggested by heavier lines around the cells immediately above the central cells. The first pericentral cell is cut off on the upper side of each central cell by a curving wall which severs the upper corner on the side of the cell away from its mate in the dichotomy (cell *a*, figs. 2, 3). Thus the upper outside pericentral cell is cut off first in each case. Next the lower outside pericentral cell is cut off in a similar manner. The middle upper pericentral cell is cut off next, followed usually by the inner lower cell. The upper inner pericentral cell is usually cut off last (fig. 3). No further cell divisions occur in vegetative portions except for the formation of rhizoids and of minute

"gland" cells. The latter are colorless and frequently arise in considerable number between the pericentral cells to which they are attached.

Rhizoids are unicellular and abundant, arising singly from the lower pericentral cells at the end farther from the margin of the frond. Mature rhizoids may be as much as  $400\ \mu$  long and  $45\ \mu$  in diameter. They are provided with lobed tips, or the tip may bear a number of short branches, each of which is terminated by a lobed expansion (fig. 13).

The cells contain great numbers of small spherical to slightly elongated chromatophores  $1.5\text{--}2.3\ \mu$  in diameter and arranged in more or less tortuous beadlike rows at the periphery of the cell. Numerous secondary pit connections develop between older cells of the thallus (fig. 10).

In its vegetative structure *Amplisiphonia* is closely similar to a much smaller plant, *Placophora binderi*, first described by AGARDH (1) in 1841 as *Amansia binderi* and later (2) established by the same author as a new genus. *Placophora binderi* grows as an epiphyte on species of *Codium*. There is but one species. It is represented in the herbarium of the University of California by no. 714 of Hauck et Richter, Phykotheke Universalis. This material was collected by Madame WEBBER-VAN BOSSE along the coast of South Africa, the type locality. As stated by HOWE (4), this plant has been reported from the coast of Peru. A bit of the African material was examined by the writer and proved to be in fruiting condition. As described and figured by FALKENBERG (3), *Placophora*, like *Amplisiphonia*, develops prostrate rounded lobes which are dorsiventral in structure and attached ventrally by numerous unicellular rhizoids which arise singly. In both plants growth is marginal by means of a row of apical cells. The number and arrangement of the pericentral cells in vegetative portions are the same in both plants. In neither are there further cell divisions after the formation of the pericentral cells, except for the formation of rhizoids and of the minute gland cells common to both plants. There seems to be no previous mention of these gland cells for *Placophora*, but examination of the material from South Africa shows that they are frequently present.

FALKENBERG (3, pl. 4, fig. 7) figures a surface view of the margin

of the thallus of *Placophora*. He does not describe the origin of the pericentral cells, but his figures would indicate that they do not arise as in *Amplisiphonia*. A single row of cells is shown as forming back of each marginal cell. This seems a very improbable arrangement in view of the fact that five pericentral cells are described as forming around each central cell as in *Amplisiphonia*. Furthermore, an examination of herbarium material of *Placophora* reveals an arrangement of cell rows very similar to that herewith described for *Amplisiphonia*, three cell rows arising back of each apical cell as seen in upper surface view. It seems probable, therefore, that there is no essential difference in the manner of origin of pericentral cells in the two plants. The forking veins do not seem to show as prominently in *Placophora* as in *Amplisiphonia*, but in vegetative features *Amplisiphonia* differs from *Placophora* chiefly in the much larger size of the frond and of the cells composing it, and in its saxicolous habit. Cells of mature portions of the thallus of *Amplisiphonia* measure  $140-180 \times 40-50 \mu$ ; those of *Placophora binderi* are scarcely more than half as large.

*Periphykon* is another epiphytic member of the Rhodomelaceae with a prostrate expanded thallus of dorsiventral structure. It was described by WEBBER-VAN BOSSE (7) from Java. It differs from *Amplisiphonia* in several vegetative characters. Four upper and two lower primary pericentral cells are formed around each central cell. The lower remain undivided but the upper divide two or three times transversely. In *Periphykon* the rhizoids arise in bundles rather than singly as in *Amplisiphonia* and *Placophora*.

#### REPRODUCTIVE STRUCTURE

The chief feature which distinguishes *Amplisiphonia* from other membranous expanded members of the Rhodomelaceae with dorsiventral structure is the fact that the tetrasporangia are borne in somewhat modified, flattened lobes of the thallus rather than in cylindrical branches as in *Placophora* and *Periphykon*. These fruiting lobes are frequently very abundant but they usually arise singly at the margin of the frond. They are at first prostrate but soon become more or less erect as the outer margin expands and they become obovate-cuneate in shape. The attachment remains narrow

(fig. 5), but the margin continues to expand, and as a result the fruiting lobes become much ruffled before the outermost tetrasporangia have matured and shed their spores. Tetrasporangia mature in marked acropetal succession in an outwardly migrating zone, and the spores are shed as soon as mature. Hence in older fruiting lobes this narrow zone of mature sporangia forms a conspicuous band near the margin (figs. 1, 5). The fruiting lobes have a rounded or more or less crenate margin. They differ from the vegetative portions of the thallus chiefly in the lack of dorsiventrality, there being three pericentral cells above and three below each central cell. This lack of dorsiventrality in fruiting portions of the thallus suggests that *Amplisiphonia* evolved from some membranous but erect form without dorsiventrality similar to *Symphycladia* or *Pollexfenia*. Vertical sections of fruiting lobes of the thallus parallel to the margin and just back of the apical cell row also show that the lack of dorsiventrality is an early and basic feature. The first pericentral cells cut off arise in a manner similar to the origin of corresponding cells in vegetative portions, but they may arise on either side of the frond (figs. 4, 7) rather than on the upper side only as in vegetative portions. Furthermore, in the case of two cell rows composing a dichotomy at the margin of the thallus, the first pericentral cell cut off from a central cell of one row may be on the same side of the thallus as the first pericentral cell cut off from a similarly placed cell in the other cell row of the dichotomy, or it may be diagonally opposite (fig. 4). Later developments occur in such a manner that the tetrasporangia appear to arise on the side of the central cell opposite that from which the first pericentral cell is cut off. It appears, moreover, that the first pericentral cell arises in a comparable position on the cells of a given cell row for a number of successive cells, and then the position of their origin may shift to the opposite side of the thallus for a similar series of cells in the cell row (fig. 12). As in vegetative portions, the second pericentral cell formed in fruiting portions is cut off directly opposite the first on the opposite side of the thallus (figs. 4, 7), and the third is cut off next to and on the same side of the thallus as the first. The fourth pericentral cell is usually cut off diagonally opposite the second, the fifth directly opposite the third, and the sixth diagonally oppo-

site the first. The order of origin may vary somewhat, the fourth and fifth cells frequently arising in reverse order.

Tetrasporangia always arise from the middle of the three pericentral cells on a given side of the thallus, and usually from the cell opposite the third (*e*, fig. 4). As already indicated, this is usually fifth in origin. Sections indicate that the third pericentral cell may occasionally give rise to tetrasporangia. The fertile pericentral cell forms the stalk cell (*e*, figs. 4, 8, 10, 12), after cutting off two cover cells (*g* and *h*, figs. 4, 8, 9, 10, 12) outwardly and a tetrasporangial cell on the side of the stalk cell toward the margin of the thallus. Hence the tetrasporangium develops between the central cell and the cover cells.

In the foregoing account it will be noted that the origin of the tetrasporangia in *Amplisiphonia* is essentially the same as that described by FALKENBERG (3), YAMANOUCHI (8), and KYLIN (5, 6) for *Polysiphonia* and other cylindrical members of the family, and as suggested by FALKENBERG for *Placophora*. It would seem that the shifting position of origin of tetrasporangia from one side of the thallus to the other (fig. 12), which usually occurs at a dichotomy, is to be understood as a modification of the spiral arrangement of the tetrasporangia common in *Polysiphonia* and other members of the family in which they arise in cylindrical branches. It must be kept in mind that in plants like *Amplisiphonia* and *Placophora* each row of central cells with its pericentral cells, arising as it does from a single apical cell, is to be considered homologous with a branch of *Polysiphonia*, for example. In this connection it is of interest to note that no suggestion of a spiral arrangement of the tetrasporangia was observed in the tetrasporic specimens of *Placophora* examined.

Tetrasporangia usually arise 5-7 cells back of the apical cells. Like the other cells of the thallus, they are uninucleate when first formed and contain numerous small, nearly spherical chromatophores. When the tetrasporangia are 25-30  $\mu$  in diameter, the nucleus divides to form four nuclei, after which cytoplasmic constrictions divide the protoplast into four tetraspores. Cytokinesis remains incomplete, however, until the tetrasporangia have increased to nearly their maximum size, 70-100  $\mu$  in diameter (fig. 9). The first division of the tetrasporangium is in a plane parallel with

the surface of the thallus. The half next to the cover cells then divides perpendicular to the first plane of division and also perpendicular to the length of the central cells. The inner half, next to the central cell, tends to divide in a plane perpendicular to the plane of division of the outer half of the sporangium (figs. 9, 11), but the plane of division of the inner half is frequently oblique or sometimes nearly parallel with the plane of division of the outer half (fig. 11). From the foregoing description it will be noted that the division of the tetrasporangium is approximately cruciate or quadrate, although KYLIN (6) states that in the Ceramiales division of the tetrasporangium is always in a tetrahedral manner. Moreover, the writer has observed a type of division of the tetrasporangium of certain species of *Pterosiphonia* and *Herposiphonia* very similar to that described for *Amplisiphonia*. It is perchance simply a modification of the tetrahedral manner of division associated with a dorsiventral or flattened thallus. The tetrasporangia apparently emerge in the usual manner between the cover cells. Efforts to find sexual plants of *Amplisiphonia* have so far proved fruitless.

### Summary

1. *Amplisiphonia* represents a new member of the Rhodomelaceae close to *Placophora* in vegetative structure. It differs from that genus in size and habit, but chiefly in the fact that the tetrasporangia are borne in modified lobes of the thallus rather than in cylindrical branches.

2. The dorsiventral thallus grows as a result of the activity of a row of marginal cells which give rise to forking rows of cells which are laterally united and which function as central cells, cutting off three pericentral cells above and two below in regular sequence.

3. Fruiting portions of the thallus lack dorsiventrality, three pericentral cells being cut off above and three below each central cell. As in a number of other members of the family, the tetrasporangia arise from certain modified pericentral cells after the latter have cut off outwardly two cover cells in each case. They divide more or less cruciately. Sexual plants are unknown.

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## STEM ANATOMY OF CHAPARRAL SHRUBS

KATHERINE S. WATKINS

(WITH FOURTEEN FIGURES)

### Introduction

The chaparral formation of southern California includes numerous species of shrubs exhibiting marked similarities in habit and leaf structure (6). This investigation was undertaken in order to determine to what extent the dominant chaparral shrubs show a corresponding resemblance in stem structure. Collections were made over a period of a year and a half from various localities throughout southern California where typical chaparral communities occur. The following species were studied: *Quercus dumosa* Nutt., *Adenostoma fasciculatum* H. & A., *Cercocarpus betuloides* Nutt., *Prunus ilicifolia* Walp., *Rhamnus californica* Esch., *R. crocea* var. *ilicifolia* Greene, *Ceanothus spinosus* Nutt., *C. divaricatus* Nutt., *Rhus laurina* Nutt., and *R. ovata* Wats. (13).

### Investigation

#### PITH

Owing to environmental conditions, growth of chaparral plants as a rule is slow (29). The pith is usually of small diameter, except in suckers and in shoots produced immediately following a fire. Specimens of *Adenostoma fasciculatum* obtained soon after a fire have an unusually large pith, as do also species of *Rhus* growing in less xerophytic situations. The pith of some species of *Ceanothus* is characteristically of larger diameter than that of other chaparral plants. In *Quercus dumosa* and the three members of the Rosaceae studied, lignification of the pith is uniformly heavy throughout, while in *Rhus* the lignification is uniform but slight. In *Rhamnus* and *Ceanothus* the peripheral cells of the pith differ from those of the central portion, both in their heavier lignification (23) and in that they remain alive while the inner cells lose their protoplasm. In many cases living cells or bands of living cells occur in the inner

portion (2). Rarely the innermost cells in *Ceanothus spinosus* escape lignification.

An abundance of stored starch occurs in the pith, especially in the smaller, more closely packed peripheral cells. Aleurone grains, crystals of calcium oxalate, tannin, or substances which are apparently glucoside-like may also occur, either associated with the starch or in separate cells. In *Rhus* small resin ducts are found in the outermost region of the pith. Small simple pits are usually abundant.

#### XYLEM

WARMING (28) states that the wood of xerophytes resembles "autumn wood"; but, with the exception of *Quercus dumosa*, which has very few vessels, all the chaparral plants studied have numerous vessels, with lumina larger than those generally ascribed to late wood. CANNON's experiments (5) show that non-irrigated desert plants have vessels more numerous and with larger lumina than those of irrigated plants. STARR (24) finds in dune plants a tendency for vessels to be more numerous in xerophytic than in mesophytic forms.

In *Adenostoma* (fig. 10), *Prunus*, *Rhamnus californica* (fig. 12), and *Rhus laurina* (fig. 14) annual rings are poorly defined, while in *Quercus* (fig. 9), *Cercocarpus* (fig. 11), *Rhamnus crocea* var. *ilicifolia*, and *Ceanothus* (fig. 13) the spring and summer wood are readily distinguishable. When present, annual rings are commonly very narrow, rarely exceeding 1 mm. in width. This condition has been shown to occur also in dune (24) and in desert plants (30). The wood is predominantly diffuse porous, tending toward ring porous in *Prunus ilicifolia*, *Rhamnus crocea* var. *ilicifolia*, and *Ceanothus spinosus*. *Quercus dumosa* shows a peculiar radially diffuse porosity (fig. 9), which is characteristic of certain other oaks as well (12). According to WEBBER (30), ring porous wood is characteristic of *Rhus ovata*; but I find only a very slight indication of ring porosity in this and other species of *Rhus*.

#### STORAGE TISSUE

The presence of abundant storage tissue in plants has been correlated with dry seasons (3). The xylem of chaparral plants con-

tains abundant storage tissue in the form of ray and diffuse parenchyma. Substitute fibers packed with starch are reported in certain desert plants (21); fibers of that nature were not observed in the chaparral plants studied by the writer. The rays are mostly uniseriate, more rarely biseriate or multiseriate. No aggregate types were found, nor were the broad rays common to many species of oaks observed in *Quercus dumosa*. The walls are always lignified, and provided with numerous pits, which in all genera studied except *Rhus* are bordered where they are in contact with the vessels. In *Quercus* both simple and bordered pits are found.

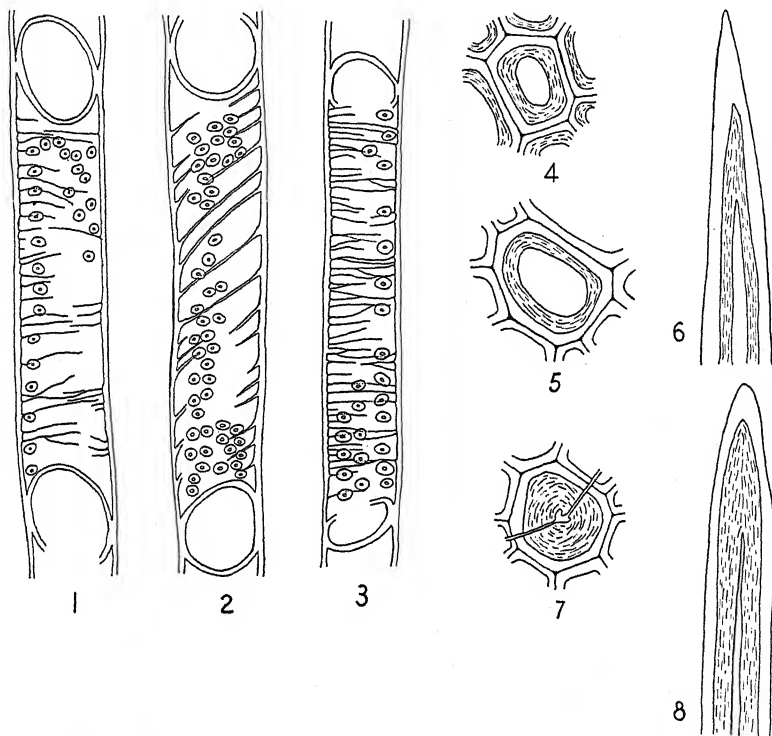
Starch grains are numerous in most of the ray cells, in *Rhus laurina* and *R. ovata* being associated with tannin, and in the Rhamnaceae and Rosaceae with glucoside-like substances. Crystals of calcium oxalate are numerous in *Quercus* and *Ceanothus spinosus*, but are rare in *Rhamnus* and other species of *Ceanothus*. As in the pith, the common storage product is starch.

Diffuse wood parenchyma is abundant in *Quercus dumosa*, *Adenostoma fasciculatum*, and *Cercocarpus betuloides*, and rare in *Prunus ilicifolia* and *Rhamnus*. In *Rhamnus* the diffuse parenchyma tends to be somewhat vasicentric. Commonly *Ceanothus* and *Rhus* have no wood parenchyma, but a few cells may be observed accompanying vessels. Calcium oxalate crystals are found in the diffuse parenchyma of *Quercus*, but are rare in *Ceanothus spinosus*. Traces of oil were noticed in *Quercus*, *Cercocarpus*, and *Rhus*, but were not sufficiently widespread to indicate importance in nutrition. Oil as a reserve material has been shown to be present in appreciable amounts in desert plants (21), even impregnating the lignified walls of the xylem and phloem elements; but in chaparral plants, as previously pointed out, starch is the common storage substance.

#### WATER-CONDUCTING TISSUES

The water-conducting tissues of chaparral plants are well developed. The lignified walls of all the plants studied have bordered pits. In *Quercus* and *Rhus* these pits are in vertical rows or more or less spirally arranged. Except in *Quercus* and *Rhus*, spiral and reticulate thickenings of the pitted vessel wall are common in all species studied (figs. 1-3). In *Rhus ovata* these spiral thickenings are rarely

present. This feature has been observed in other chaparral plants and in many desert plants (30). Perforations of the end walls are simple. SOLREDER (23) reports scalariform perforations in all the species of *Quercus* studied; I find none in *Quercus dumosa*.



FIGS. 1-8.—Figs. 1-3, vessel elements: 1, *Adenostoma fasciculatum*; 2, *Prunus ilicifolia*; 3, *Ceanothus spinosus*;  $\times 400$ . Figs. 4-8, mucilaginous fibers: 4, from xylem of *Rhus laurina*; 5, from xylem of *Rhamnus californica*; 6, from xylem of *Ceanothus divaricatus*; 7, 8, from phloem of *Quercus dumosa*;  $\times 930$ .

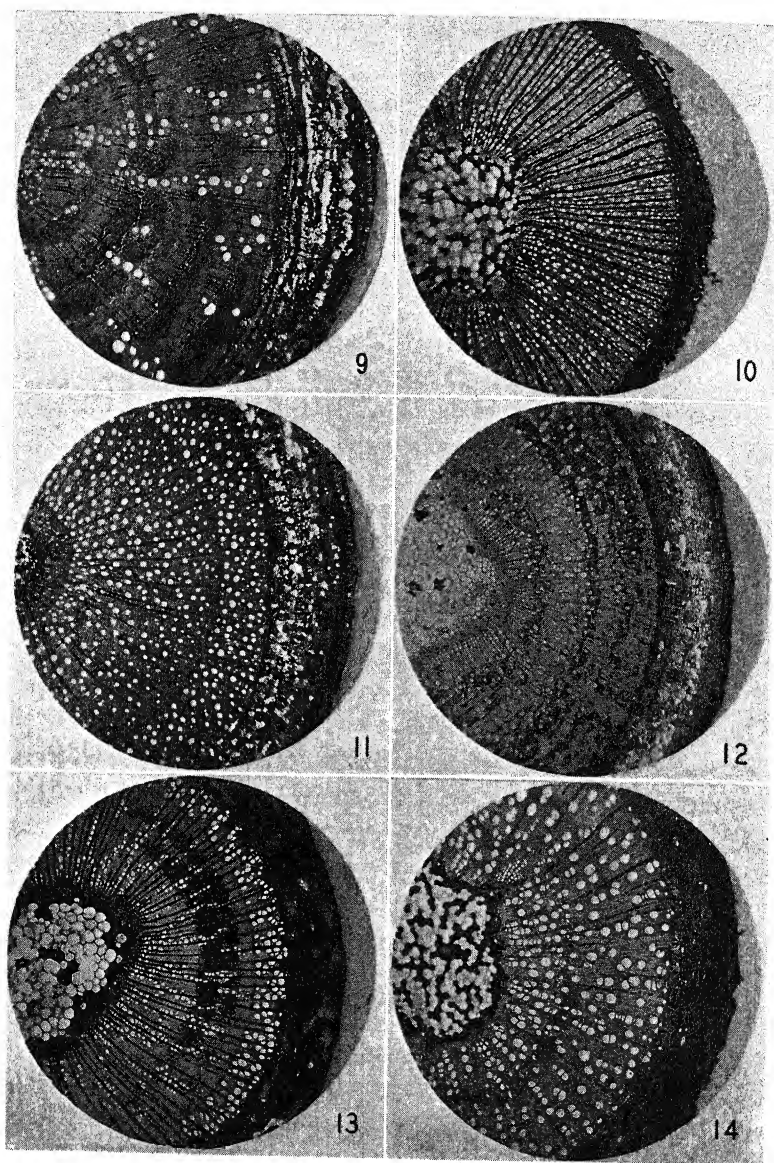
The average diameter of the lumina is variable, being largest in the vessels of *Quercus* and *Rhus* and smallest in those of *Adenostoma* and some species of *Ceanothus*. The vessel elements are comparatively short, commonly reaching a maximum length in *Quercus* of  $480\ \mu$ , and a minimum length of  $75\ \mu$  in *Adenostoma fasciculatum*. WEBBER (30) reports a shorter average length for a number of

desert forms. Gummosis occurs in *Quercus*, *Adenostoma*, and *Prunus*. In nearly all the plants studied a few of the vessel elements may have the lumina completely filled with gum. The tyloses commonly present in the vessels of *Quercus* were only rarely observed in *Q. dumosa*.

Tracheids are commonly absent in plants with a complex stem xylem having the functions of support and conduction definitely dissociated (8). In *Q. dumosa*, however, radially arranged bands of heavy-walled, border-pitted tracheids are found accompanying the vessels, apparently connecting different parts of the secondary woody cylinder. JEFFREY (12) reports groups of fiber tracheids lateral to the bands of tracheids in *Q. rubra*; I did not find these mechanical elements in *Q. dumosa*.

#### MECHANICAL TISSUES

The extensive development of mechanical tissue is generally accepted as a xerophytic character. This tissue in chaparral plants presents a number of peculiar features in addition to the heavy lignification and generally small lumina of the numerous fibers. With the exception of the three genera of the Rosaceae studied, irregularly scattered groups of fibers having only partly lignified walls were found in all cases. The distribution of these fibers is indicated in figures 9, 12, 13, and 14 by the darker stained areas. A number of investigators (2, 8, 10, 12, 25) report this type of fibrous element in plants of widely separated systematic affinities. The inner portion of the wall is apparently of a gelatinous consistency, and therefore is excluded from the process of lignification as outlined by ANDERSON (1) and others (4, 11). JEFFREY (12), EAMES and MACDANIELS (8), and a number of others find this type of fiber in the wood of *Quercus rubra* (8), in other species of oaks, in the Leguminosae (12), in *Morus alba*, *Betula alba*, *Diospyros virginiana*, *Eucalyptus cordata*, etc. (2). These investigators designate the fiber as "mucilaginous." LECLERC DU SABLON (15, 16) and SCHELLENBERG (10, p. 661) believe that this gelatinous layer is made up of hemicelluloses which are deposited late in the season and are utilized by the plant the following spring. No evidence for this use of the inner



FIGS. 9-14.—Cross sections: Fig. 9, *Quercus dumosa*, 7-year-old stem. Fig. 10, *Adenostoma fasciculatum*, 3-year-old stem. Fig. 11, *Cercocarpus betuloides*, 3-year-old stem. Fig. 12, *Rhamnus californica*, 2-year-old stem. Fig. 13, *Ceanothus divaricatus*, 2-year-old stem. Fig. 14, *Rhus laurina*, 2-year-old stem.  $\times 20$ .

wall layers of these fibers, however, was found in the chaparral plants studied.

According to MANGIN (17), the several varieties of gums and mucilages give reactions depending on their origin from cellulose, callose, or pectic substances. A mucilage derived from a pectic or allied substance gives a bright pink-red color when treated with ruthenium red. Although this test is not specific for pectic substances, it indicates the presence of any polysaccharides containing glycuronic or galacturonic acid; for example, pectic compounds, gums, mucilages, or hemicelluloses (14).

In addition to giving a positive test with ruthenium red, the inner layers of the so-called mucilaginous fibers in the chaparral stems give a positive test for cellulose with chlor-zinc-iodide and other reagents (figs. 4, 5, 6). Use of the polarizing microscope offers a further check, indicating the presence of cellulose and some non-crystalline substance. This substance, as has been shown, is probably a mucilage derived from a pectic or allied compound. I have been unable to find any reference to the distribution of mucilaginous fibers in *Rhamnus*, *Ceanothus*, or *Rhus*. They have been found in a number of species of *Quercus*, but no mention of *Q. dumosa* has been made in this connection.

Typical libriform fibers also are present in the wood. These, as well as the mucilaginous elements, are provided with bordered or simple pits and are heavily lignified. No substitute fibers, such as those found by SCOTT (22) to form the bulk of the wood of *Parinsonia aculeata*, were observed in the chaparral plants here studied.

#### PHLOEM

The cell walls of the sieve tubes, companion cells, parenchyma, and ray cells are composed of cellulose and pectic compounds. In *Quercus dumosa* the phloem ray cells become lignified, even those only one cell removed from the cambial initials. Compound crystals of calcium oxalate are nearly always found in the phloem. Solitary crystals are also present sometimes, as in *Quercus* and *Rhamnus*, forming in the parenchyma surrounding the groups of fibers to make a continuous sheath of crystallogenous cells. Chloroplasts occur in most cases, at least in the outer layers. A few starch grains are also

generally present. In *Adenostoma fasciculatum* traces of oil occur in the parenchyma. Tannin, frequently in elongated sacs, is common in the phloem of *Rhus*, and may also occur in the ray initials. The phloem parenchyma of *Quercus* and *Prunus* may contain small amounts of tannin. A characteristic feature of all the species of *Rhus* is the presence of large resin ducts in the outer region of the phloem, and of small ducts in the inner region.

The glucoside xanthorhamnin is known to be present in the parenchyma and phloem ray cells of *Rhamnus* (7). The writer has found similar substances in *Ceanothus* which presumably are of the same nature as the glucoside of *Rhamnus* (19, 27, 31). Substances which may possibly be related to l-amygdalin are found in the phloem ray cells of *Adenostoma*, and in the phloem and phloem parenchyma and rays of *Cercocarpus* and *Prunus*. *Prunus ilicifolia* may contain traces of prunasin, a glucoside reported present in *Prunus serotina*, *P. macrophylla*, and *Photinia serrulata* (27).

Those plants which have been referred to as having mucilaginous fibers in the xylem may have the same type of fiber in the phloem, as in *Quercus*. When present, the mucilaginous portion of the phloem fiber is more extensive than that of the xylem fiber, further reducing the size of the lumen, often almost to the point of complete disappearance (figs. 7, 8). The phloem of *Quercus dumosa*, *Cercocarpus betuloides*, and *Rhamnus* is marked by numerous groups of fibers. In *Cercocarpus* these form an almost continuous layer in the youngest region; in *Quercus* they are found in definite tangential bands accompanied by scattered stone cells; in *Rhamnus* they are surrounded by parenchyma containing crystals of calcium oxalate. *Prunus ilicifolia* and *Ceanothus* have only a few lignified fibers, occurring principally in the older region. *Adenostoma fasciculatum*, *Rhus laurina*, and *R. ovata* commonly have no mechanical tissue in the phloem.

#### PERICYCLE

The pericycle of chaparral plants is similar to that of other woody plants. *Quercus dumosa* and *Adenostoma fasciculatum* are the only two of the shrubs studied in which the mechanical tissues of the

pericycle form a continuous cylinder, consisting of groups of fibers united by stone cells. In *Quercus* these fibers are mucilaginous and in *Adenostoma* they are lignified. In *Cercocarpus*, *Prunus*, *Rhamnus*, and *Ceanothus* isolated groups of fibers are characteristic, with stone cells only rarely present. The walls of these fibers are lignified in *Cercocarpus* and *Prunus*, mucilaginous in *Rhamnus*, and unmodified in *Ceanothus*. The parenchyma between these isolated groups contains scattered chloroplasts and starch grains in *Cercocarpus* and *Prunus*, and is filled with a yellow-brown, glucoside-like substance in *Rhamnus* and *Ceanothus*. The pericycle of *Rhus* consists of arc-shaped groups of mucilaginous fibers inclosing the large resin ducts of the outer phloem.

#### CORTEX

Collenchyma is found in the cortex of all the plants studied, with the exception of *Ceanothus* and *Rhus*. Both collenchyma and parenchyma contain chloroplasts. Scattered stone cells are found in *Quercus*, *Cercocarpus*, and *Rhus ovata*, but sclerenchyma is rarely present in the cortical regions of the other species. The inner cortical cells of *Rhamnus crocea* var. *ilicifolia* and of *Ceanothus* are much larger and thinner walled than the cells of the outer cortex, and are filled with a yellow-brown substance. This substance is also present in other cells of the cortex, pericycle, phloem, xylem rays, and pith of *Rhamnus* and *Ceanothus*.

Throughout this discussion, this substance has been referred to as a glucoside or a glucoside-like material, rather than as a mucilage (23) or a resin (31), since its reaction with potassium hydroxide to give a red-brown or purplish brown color is that reported by TUNNMANN and ROSENTHALER (27) and others (7, 31) for the glucosides xanthorhamnin and "rhamnokysid" (27) found in several species of *Rhamnus*. While the presence of glucosides in *Ceanothus* is not indicated by any of these investigators, it is very probable that the yellow-brown substances found in the cortex and elsewhere are similar to those of *Rhamnus*. *Adenostoma fasciculatum*, *Cercocarpus betuloides*, and *Prunus ilicifolia* contain a brownish or pinkish substance in the cortex and in other parts of the stem. Here, however, the ma-

terial is less abundant and gives no color reaction with potassium hydroxide other than a slight bleaching. Apparently this substance is neither mucilaginous nor resinous, but is more nearly similar to the glucosides amygdalin, prunasin, and phlorizin described for the Rosaceae (7, 9, 26, 31). Further investigation is necessary to show definitely that these substances present in the stems of chaparral plants are glucosides, and also to determine whether they are actually of a mucilaginous or of a resinous character. Large amounts of pyrocatechol tannin are present in the cortical parenchyma and collenchyma of *Rhus* (18), and traces of it occur in the cortical parenchyma and collenchyma of *Quercus*.

Extensive cork formation is common in chaparral plants, as well as in other plants growing under dry conditions (10, 20, 25). In all cases except *Adenostoma*, where the phellogen arises in the phloem, the periderm is subepidermal. In a few of the stems of *Cercocarpus* and *Ceanothus spinosus*, a layer of cork cells was found inclosing one or two small groups of pericyclic fibers; but whether this condition was due to injury or to the initiation of a phellogen deeper in the stem was not clear. Pyrocatechol tannin is present in the cork and phelloderm cells of *Quercus* and *Rhus*, while glucoside-like substances appear in the periderm of the other genera.

### Summary

1. A comparative study of the stem anatomy of ten species, dominant in the chaparral formation of southern California, is presented.
2. The pith is lignified and generally of small diameter, containing abundant starch grains and small amounts of other ergastic substances.
3. The wood is predominantly diffuse porous, and the annual rings, when present, are narrow, rarely exceeding 1 mm. in width.
4. Reserve food is abundant in the xylem, chiefly in the form of starch.
5. Pitted vessels are numerous, mostly with spiral or reticulate thickenings on their lateral walls.
6. With the exception of the members of the Rosaceae, the presence of mucilaginous fibers in the xylem is a common feature of all

the plants studied. In *Quercus dumosa* mucilaginous fibers are found in the phloem as well.

7. Tannin is present in the phloem and cortex of *Quercus* and *Rhus*, and glucoside-like substances in the other genera.

This investigation was carried on under the direction of Professor ARTHUR W. HAUPT, to whom, as well as to Professor FLORA M. SCOTT, the writer is indebted for helpful suggestions and criticisms.

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# CHEMICAL DETERMINATION OF ETHYLENE IN THE EMANATIONS FROM APPLES AND PEARS<sup>1</sup>

ELMER HANSEN AND BERT E. CHRISTENSEN

## Introduction

In connection with physiological investigations relating to the natural occurrence of ethylene in fruits, it has been considered necessary to develop an accurate and rapid chemical method for determining the small amounts of this gas evolved from the fruits during ripening and storage. Although ethylene has been chemically identified as a constituent of the volatiles from certain fruits (2, 4), the procedures used are not applicable to the quantitative estimation of this gas as produced by the small experimental lots of fruit commonly used in physiological investigations. For this purpose, a micro-method based upon the bromination procedure previously described by CHRISTENSEN *et al.* (1) for determining the ethylene contained in plant tissues was adopted. In using this method for the determination of the ethylene evolved from apples and pears, it was necessary to ascertain whether other unsaturated hydrocarbon gases, such as acetylene, propylene, and butylene, were present in amounts sufficient to interfere with the bromination procedure used.

## Analytical procedure

### COLLECTION OF ETHYLENE

In order to build up a concentration of ethylene that could be determined by the micro-reagent used (0.001-0.1 ml. per 35 ml. of gas sample), approximately 1.5-2 kg. of fruit is inclosed for a definite period of time in a desiccator of known capacity. The fruit is supported on a false bottom, permitting the use of a 15 per cent potassium hydroxide solution below to absorb the carbon dioxide evolved.

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Atmospheric concentration of oxygen is maintained by the addition of pure oxygen from a constant water level siphon (3). After standing at the desired temperature for a definite time (usually 48 hours), a 35-ml. gas sample is withdrawn and collected over mercury in a Hempel burette. The desiccator is then opened, thoroughly rinsed out, recharged with KOH solution, and reattached to the oxygen supply. The temperature and the barometric pressure at time of sampling should be recorded.

In using this procedure the fruit is subjected to the influence of the ethylene produced during the period of confinement. Cognizance of this fact should be taken when interpreting the data obtained.

#### DETERMINATION OF ETHYLENE

The procedure and apparatus used in purifying the gas sample and in determining the ethylene contained have been fully described elsewhere (1). For convenience, the essential details of the analytical method are given here.

To the burette containing the gas sample is added 1 ml. of 2.5 per cent ammonium hydroxide solution, over which the vapors are allowed to remain for 15 minutes to absorb acetaldehyde. To remove traces of any other interfering substances, the gases are passed slowly through a small Desicchlora tube (to remove excess ammonia vapor), and then through a copper coil (2 mm. I.D.  $\times$  100 cm.) immersed in a dry ice-ether mixture contained in a Dewar flask. The purified gases are collected over mercury in a Hempel burette, where they are stored until ready for analysis.

Bromination of ethylene in the purified gas sample is carried out in a reaction flask constructed from a 50 ml. Erlenmeyer fitted with a 12/30 standard taper and a capillary stopcock. The flask is charged with 5.00 ml. of 0.0025 potassium bromate, 0.5 ml. of 6 N sulphuric acid, and then partially evacuated. The gas sample and 1 ml. of N/10 potassium bromide are then introduced without breaking the vacuum. The mixture is shaken for 15 minutes on a mechanical shaker, after which 1 ml. of N/10 potassium iodide is introduced by means of the residual vacuum. The iodine liberated is titrated with 0.0025 N sodium thiosulphate from a microburette. The amount of

potassium bromate used is determined by the difference between the blank run (using air) and the actual determination. One ml. of 0.0025 N potassium bromate is equivalent to 0.028 ml. ethylene N.P.T. Since the volumes of the aliquot and the desiccator are known, the total amount of ethylene produced can readily be calculated. A correction must be made for the volume of gas remaining in the Desicchlora tube and the copper coil. In the apparatus used this amounted to 5.3 ml.

### Tests for unsaturated hydrocarbon gases other than ethylene

Although reactive vapors such as acetaldehyde were removed prior to analysis, unsaturated hydrocarbon gases such as acetylene, propylene, and butylene may have been present in amounts sufficiently great to interfere with the determination of ethylene. To ascertain whether this were possible, the volatiles produced by apples and pears were treated with specific absorbents for acetylene, propylene, butylene, and ethylene. To determine the absence or presence of these gases, quantitative determinations of the unsaturated hydrocarbons contained before and after treatment were made. No procedures were used to remove carbon monoxide, since this gas would not be brominated, even if present.

The gases were collected from approximately 2 kg. each of Ortley apples and Bartlett pears inclosed in desiccators as described. Prior to analyses, 250-ml. gas samples were withdrawn from the containers and collected in a pipette over mercury. From these samples 35-ml. aliquots were taken for the solubility tests.

For removing acetylene from the gas sample, a solution of 20 per cent mercuric cyanide in 2 N sodium hydroxide was used. According to TREADWELL and HALL (5), this reagent removes acetylene but not ethylene from gas mixtures. Ten ml. of the mercuric cyanide solutions was placed over mercury in a Hempel burette. Approximately 35 ml. of the gas sample was then introduced and allowed to react with the reagent for 30 minutes, with occasional raising and lowering of the leveling bulb to insure complete absorption. The gas sample was then transferred to another burette containing mercury, and the unsaturates present determined. From the results

obtained (table 1) it is apparent that the unsaturates contained in the gases from both apples and pears are not soluble in mercuric cyanide solution.

For removing propylene and butylene, a solution of 87 per cent sulphuric acid was used. According to TROPSCH and MATTOX (6), both these gases are readily soluble in this reagent, but ethylene is not appreciably absorbed if present in concentrations less than 20 per

TABLE 1  
SOLUBILITY OF UNSATURATED GASES PRODUCED BY  
APPLES AND PEARS IN VARIOUS REAGENTS

REAGENT USED	ML. 0.0025 N KBrO <sub>3</sub> USED PER 100 ML. GAS SAMPLE		PERCENTAGE ABSORBED (CALCULAT- ED AS ETHYLENE)	
	BEFORE ABSORPTION	AFTER ABSORPTION		
	BARTLETT PEAR			
	Mercuric cyanide.....	6.40	6.47	None
	Sulphuric acid (87%).....	6.40	6.34	0.93
	Mercuric nitrate.....	3.88	0.33	91.67
	ORTLEY APPLE			
	Mercuric cyanide.....	4.05	4.14	None
	Sulphuric acid (87%).....	4.05	4.22	None
	Mercuric nitrate.....	5.84	0.00	100.0

cent. To the burette was added 5 ml. of this solution, then 35 ml. of the gas sample was introduced and allowed to react for 3-5 minutes. The unsaturates were then determined as formerly. From the results obtained, it is apparent that the gases are not appreciably soluble in this reagent. Only 0.93 per cent of the unsaturates from the pears were absorbed, while none were removed from the apple sample.

To absorb ethylene, a solution of 20 per cent mercuric nitrate in 2 N nitric acid saturated with sodium nitrate was prepared. This reagent is used by TREADWELL and HALL (5) to remove ethylene

from gaseous mixtures. To the burette was added 5 ml. of this solution, then the gas sample was introduced and allowed to react for 30 minutes. The data show that the unsaturated gases from both apples and pears are highly soluble in mercuric nitrate solution. Approximately 92 per cent of the unsaturates in the gas sample from the pears was absorbed by this reagent, while that from the apples was found to be completely soluble.

It is apparent that ethylene is the active gas evolved from the apples and pears used in these experiments. If similar hydrocarbons such as propylene, butylene, and acetylene are produced, they must occur only in small amounts that would not interfere with the bromination method used for determining ethylene.

#### Determination of ethylene produced by apples and pears

To ascertain whether the method is applicable for the purpose intended, the amounts of ethylene produced by apples and pears at

TABLE 2  
AMOUNTS OF ETHYLENE PRODUCED BY APPLES  
AND PEARS DURING RIPENING

DAYS RIPENED	ML. ETHYLENE PER KG.-HOUR			
	ANJOU PEAR 65° F.	BARTLETT PEAR 42° F.	BARTLETT PEAR 65° F.	GRAVENSTEIN APPLE 65° F.
2.....	0.003	0.113	0.222	0.100
4.....	0.003	0.113	0.251	0.135
6.....	0.000	0.101	0.055	0.220
8.....	0.000			
9.....		0.135	0.000	0.280
10.....	0.012			
12.....	0.021			
14.....	0.030			

various stages of maturity and ripeness have been determined. The Anjou pears used were taken at time of picking, while the Bartletts had been held previously for ten weeks in cold storage. The Gravenstein apples had not been subjected to cold storage but were beginning to ripen at the start of the experiment. All samples of fruit

were held at a temperature of 65° F. during the course of the experiment, with the exception of one lot of Bartlett pears which was held at 42° F. The data are shown in table 2.

It is apparent from table 2 that the amounts of ethylene produced by both Bartlett pears and Gravenstein apples are well within the range determinable by the method described. The production of ethylene by Bartlett pears held at 42° F. remained fairly constant during the course of the experiment, while the ethylene produced by those ripened at 65° F. decreased rapidly and finally dropped below 0.001 ml. per kg.-hour when the fruit had reached an advanced stage of breakdown. The amounts produced by the Gravenstein apples increased steadily during ripening. The production of ethylene by the Anjou pears was barely within the analytical range during the early stages of ripening, but could be determined readily thereafter.

It is interesting to compare the values obtained with the data reported by GANE (2), who passed the emanations from 60 pounds of Worcester Pearmain apples for four weeks through pure bromine. By this procedure was obtained 0.85 gm. of oil, which on fractional distillation yielded 0.65 gm. boiling below 140° C. (b.p. ethylene dibromide 131.7° C.). This amount of oil as ethylene dibromide is equivalent to approximately 97 ml. of ethylene, which would be in the order of 0.005 ml. per kg.-hour. This value compares favorably with the amount of ethylene evolved by Anjou pears during the early stages of ripening but is considerably lower than that produced by Gravenstein apples.<sup>2</sup>

### Summary

1. A chemical method based upon a bromination procedure has been adapted for determining the ethylene contained in the emanations from apples and pears.
2. Solubility tests have shown that ethylene is the active gas

<sup>2</sup> The following publications came to the attention of the writers after this paper had been prepared for publication.

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evolved from the fruits used. Similar unsaturated hydrocarbon gases such as acetylene, propylene, and butylene were not found to be present in amounts that could be detected by the bromination procedure used.

3. The amounts of ethylene produced by several varieties of apples and pears during ripening were determined and found to be within a range of  $<0.001$  to  $0.280$  ml. per kg.-hour.

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# COMPARISON OF GROWTH RESPONSES INDUCED IN PLANTS BY NAPHTHALENE ACETAMIDE AND NAPHTHALENE ACETIC ACID

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(WITH TEN FIGURES)

## Introduction

Some compounds capable of causing cell elongation in the pea test (7) have the following structural characteristics: (a) a ring system as the nucleus; (b) a double bond in the ring; (c) a side chain; (d) a

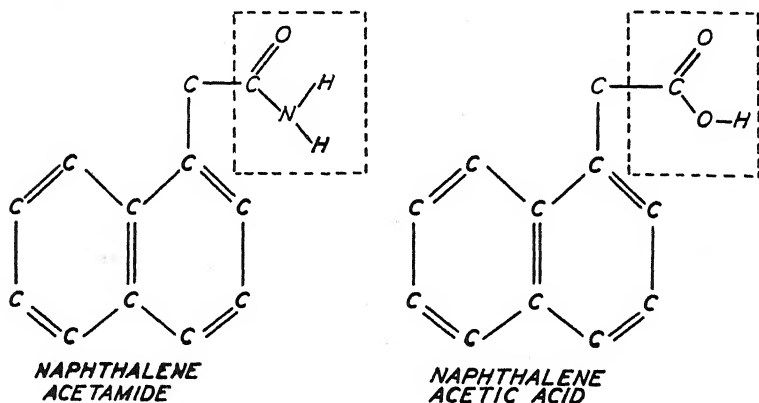


FIG. 1.—Difference in structural formulae of alpha naphthalene acetamide and alpha naphthalene acetic acid.

carboxyl group, or a structure readily converted into a carboxyl group, which is at least one carbon atom removed from the ring; and (e) a particular space relationship between the ring and the carboxyl group (4). Alpha naphthalene acetamide and alpha naphthalene acetic acid possess some of these characteristics in common, both being capable of inducing growth responses; but naphthalene acetamide

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does not possess a side chain with a carboxyl group. Furthermore each compound induces some specifically characteristic responses while others are common to both substances.

It has been suggested that some plants are capable of converting certain compounds (such as tryptamine, tryptophane, naphthalene aceto nitrile) that have no carboxyl side chains to their corresponding oxidized or hydrolyzed forms which contain a carboxyl group in the side chain and are then capable of inducing cell elongation (7, 8). Alpha naphthalene acetamide induces several responses in plants in addition to cell elongation; for example, increased secondary thickening, lignification of cell walls, root formation, delay in the formation of abscission layers (5), and parthenocarp (1). In view of the chemical similarity of naphthalene acetamide and naphthalene acetic acid (fig. 1), experiments are reported here which were designed to compare some of the responses resulting from the application of these two naphthalene compounds to several species of plants.

### Investigation

#### PEA TEST

Naphthalene acetamide and naphthalene acetic acid (6) were active in causing cell elongation. The former showed activity at concentrations of 100 mg. per liter. The same concentration of the latter compound was toxic, as shown by flaccidity of the tissue. This test was repeated several times with the same results. From these data it appears that naphthalene acetamide is an auxin; that is, a substance causing growth promotion by cell elongation.

#### AVENA TEST

The standard *Avena* test (8) for growth activity of naphthalene acetic acid showed negative curvatures. These have been observed by WENT and THIMANN (8). Under the same test conditions (250 gamma per liter), naphthalene acetamide resulted in no growth curvature during the standard 90 minutes, or even after 24 hours. Negative curvatures resulted when applications were applied unilaterally at very much higher concentrations in lanolin (fig. 2). In the standard *Avena* test the concentration was possibly too low and

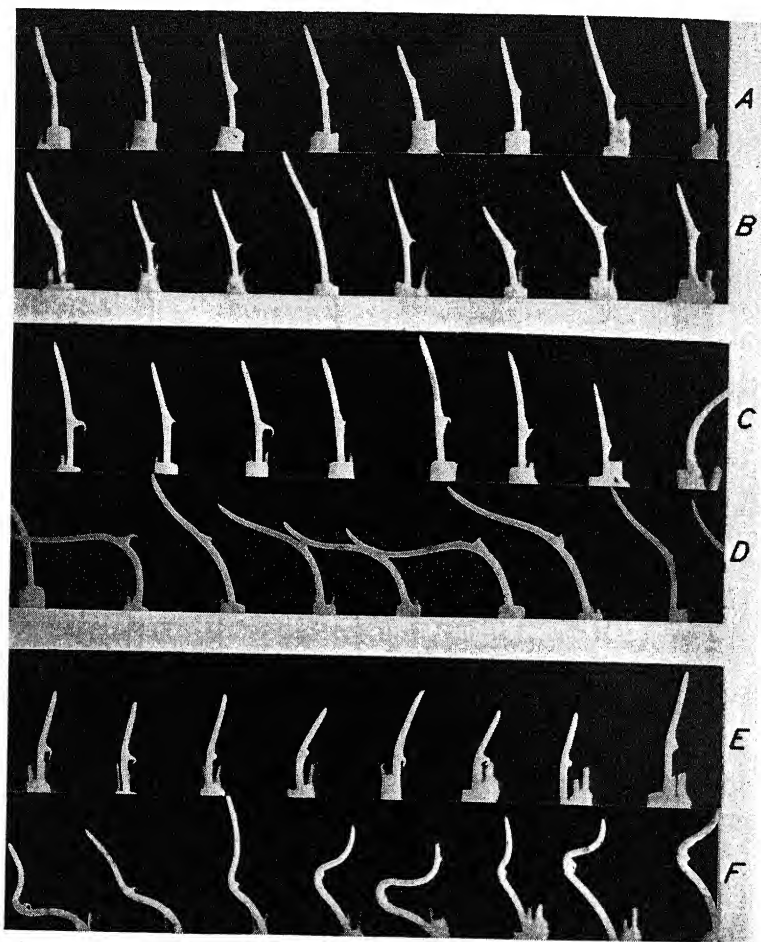


FIG. 2.—Bending responses following unilateral applications of lanolin mixtures. *A*, 0.02 per cent naphthalene acetamide after 2 hours; *B*, after 24 hours; *C*, 0.02 per cent naphthalene acetic acid after 2 hours; *D*, after 24 hours; *E*, 2 per cent naphthalene acetic acid after 2 hours; *F*, after 24 hours.

there were insufficient amounts of the acetamide or some derivative from it to act as a growth promoting substance. These experiments have been repeated three times with similar results.

### BENDING RESPONSES

Naphthalene acetamide and naphthalene acetic acid in lanolin applied unilaterally to rapidly growing stems caused either negative or positive bending. The response varied depending on the concen-

TABLE 1

POSITIVE OR NEGATIVE CURVATURES\* INDUCED BY UNILATERAL APPLICATIONS TO STEMS OF 2 PER CENT MIXTURE OF LANOLIN AND NAPHTHALENE ACETIC ACID, NAPHTHALENE ACETAMIDE, AND INDOLEACETIC ACID

PLANT	EXPERIMENTAL CONDITIONS	HOURS AFTER APPLICATION								
		NAPHTHALENE ACETIC ACID			NAPHTHALENE ACETAMIDE			INDOLEACETIC ACID		
		3	8	24	3	8	24	3	8	24
Pea.....	Etiolated	—	+	+	o	—	—	—	....	—
	Light grown	—	....	+	o	o	o	—	—	—
Kidney bean.....	Etiolated	—	+	+	o	—	—	—	—	—
	Light grown	—	—	—	o	o	—	—	—	—
Petunia.....	Light grown	....	....	+	....	....	—	....	....	—
Soybean.....	Light grown	—	—	+	o	o	o	—	—	—
Tomato.....	Light grown	—	+	+	o	o	o	—	—	—
Four o'clock.....	Light grown	....	....	+	....	....	o	....	....	—
Avena coleoptile.....	Etiolated	+	+	+	—	—	—	—	—	—

\* Bending toward the side to which mixture was applied was designated positive.

tration of the mixture, the species, and the conditions under which the plants were grown. The results are summarized in table 1 and figures 3 and 4. Each observation was based on fifteen to thirty plants and repeated twice with the same results. With applications of 2 per cent naphthalene acetic acid to stems of etiolated kidney bean seedlings, and seedlings of tomato, garden pea, and soybean grown in the light, negative curvatures initiated during the first 3 hours were subsequently followed by positive curvatures. This effect may have been the result of an increasing concentration of naphthalene acetic acid inside the plant, as inward diffusion from the

surface continued. That low concentrations accelerated while higher concentrations inhibited cell elongation is also evident, in that a 2 per cent naphthalene acetic acid paste induced strong ( $19^{\circ}$ ) positive curvatures on *Avena* coleoptiles in 2 hours, while 0.02 per cent

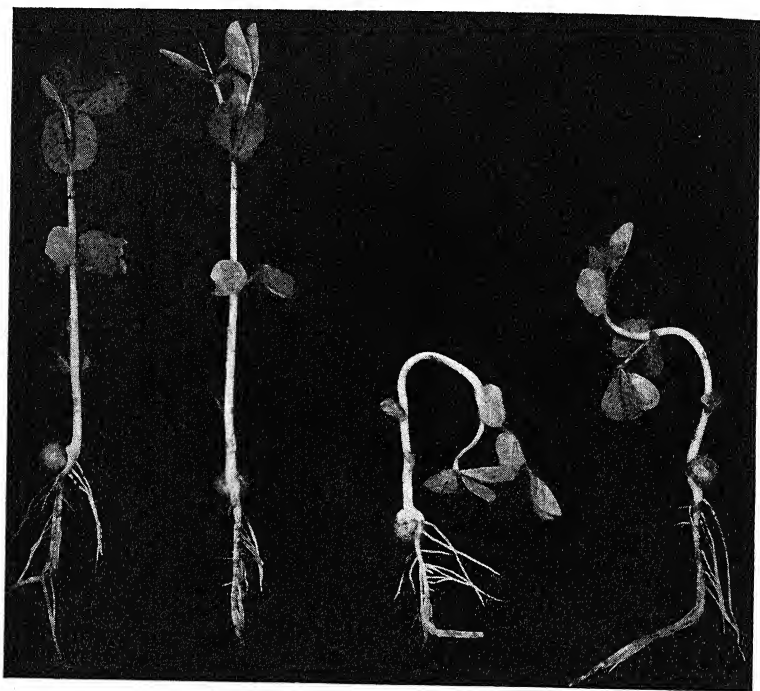


FIG. 3.—Stem curvatures of pea seedlings 24 hours after unilateral applications of 2 per cent mixtures. Left to right: pure lanolin; naphthalene acetamide; naphthalene acetic acid (positive curvature); and indoleacetic acid (negative). Plants grown in light.

applications induced  $17^{\circ}$  negative curvatures. Two per cent applications of the same substance caused positive curvatures of tomato stems. Positive curvatures of sweet pea stems resulting from applications of 1.5 per cent lanolin mixtures of naphthalene acetic acid and negative bending from 1 per cent applications have been reported by ZIMMERMAN and WILCOXON (9).

Only negative curvatures were observed following application of 2 per cent lanolin mixtures of naphthalene acetamide. These were

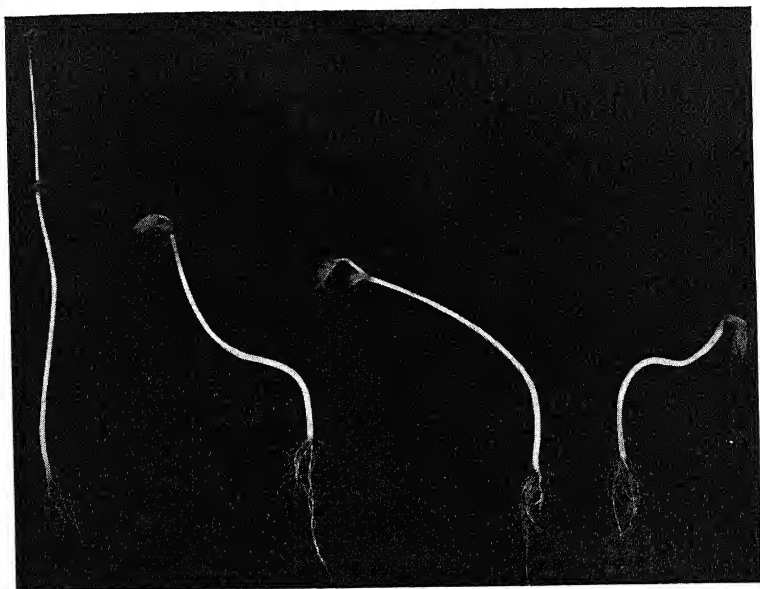


FIG. 4.—Stem curvatures of etiolated kidney bean plants treated unilaterally with 2 per cent lanolin mixtures. Left to right: pure lanolin; indoleacetic acid (negative curvature); naphthalene acetamide; and naphthalene acetic acid (positive).



FIG. 5.—Response of petunia leaves to naphthalene acetamide (left) as compared with naphthalene acetic acid; 2 per cent lanolin mixtures applied to stems.

usually delayed for 12-24 hours, suggesting that possibly during this time the compound was converted slowly to a growth promoting substance, which then induced cell elongation.

In response to unilateral applications of a 2 per cent lanolin mixture of naphthalene acetamide the uppermost three or four leaf blades of petunia plants curled markedly downward (fig. 5). The stem showed a slight negative curvature distributed throughout its length. The leaf curling response did not occur with applications of naphthalene acetic acid, or indoleacetic acid. As the leaves were not flaccid the curling was apparently the result of differential growth of the upper and lower surfaces.

#### EMULSION SPRAYS

To compare the effects of spray applications of naphthalene acetamide with those resulting from the use of other sprays containing naphthalene acetic acid, kidney bean plants were grown in sand with nutrient solution. The plants were selected for uniformity when 4-6 inches high and sprayed with emulsions (3) containing various amounts of the acetamide and acid respectively. Each treatment was applied to approximately 150 individual plants previously distributed at random throughout the total used.

One-half of the plants of each treatment were harvested 5 days and the remainder 10 days after treatment. They were washed free from sand and divided into roots, hypocotyls, primary leaves, first internodes, and tops (all parts above the second node). These samples were weighed, then dried in a well ventilated oven and the dry weight recorded.

Several of the responses that resulted from use of the acetamide were similar to those resulting from use of the acid. On the other hand, dissimilar responses were noted also. Thus in high concentrations (25-625 mg. per liter) both naphthalene compounds decreased internodal elongation. This response was first evident within 48 hours after treatment. Five days after treatment the heights of plants sprayed with the acid were 28, 44, and 49 per cent less than that of controls when 25, 125, and 625 mg. of acid per liter were used respectively. Leaf expansion was likewise inhibited by both compounds. Treated leaves were lighter green in color and apparently thicker

than those of controls. The margins curled evenly downward within a few days following treatment and remained permanently in this position. This effect was most pronounced in the case of the acetamide treatment.

Another response common to both naphthalene compounds was root initiation on the stems. This response was marked in the case of the most concentrated acid spray, root primordia being evident on the hypocotyl and first internode of all the plants. Roots were evident on the upper part of the hypocotyls on a few of the plants treated with the most concentrated spray of acetamide. It was also observed that the abscission of cotyledons from plants treated with both naphthalene compounds occurred several days later than from control plants.

Among dissimilar responses the most striking was the difference in the amount of bending induced by the two compounds. Within 2 hours following treatment, plants sprayed with 25, 125, and 625 mg. of naphthalene acetic acid per liter showed definite and uniform nastic response, the stems curving away from the direction of spraying. The intensity of this response varied in proportion to the concentration of spray used. Twenty-four hours after treatment plants sprayed with 25 mg. per liter had recovered and were erect, while curvatures induced with stronger concentrations were permanent throughout the remainder of the experiment. In contrast to this, the use of the acetamide resulted in no apparent curvature of stems.

Low concentrations (5 and 25 mg. per liter) of naphthalene acetamide resulted in a slight increase of growth of the tops of bean plants, as shown by dry weight measurements (table 2). Comparable concentrations of naphthalene acetic spray had no appreciable effect. Further difference in responses was observed in that 625 mg. of acetamide per liter caused approximately 48 per cent increase in the weight of roots over that of control plants, while the acid had no appreciable effect. Roots of plants sprayed with this concentration of acetamide were more fibrous than those sprayed with a comparable concentration of the acid or those of control plants.

In summary, the more concentrated sprays of acetamide stimulated root growth, inhibited top growth, and stimulated the production of relatively few root primordia from the stem. Less concen-

trated sprays resulted in increased top growth. On the other hand, the acid inhibited both root and top growth and in the concentrated sprays induced marked curvature of stems with formation of numerous root primordia.

TABLE 2

EFFECT OF EMULSION SPRAYS CONTAINING DIFFERENT CONCENTRATIONS OF NAPHTHALENE ACETAMIDE AND NAPHTHALENE ACETIC ACID. FIGURES REPRESENT GRAMS DRY WEIGHT OF PARTS OF 100 BEAN PLANTS HARVESTED 10 DAYS FOLLOWING TREATMENT

PLANT PART	BLANK	LANOLIN EMULSION ONLY	MILLIGRAMS PER LITER			
			5	25	125	625
Tips..... Primary leaves..... First internode..... Hypocotyl..... Roots.....  Total.....	65.0 25.4 5.6 9.8 15.1  120.9	64.9 24.7 5.6 9.9 16.3  121.4	NAPHTHALENE ACETAMIDE			
			68.1	75.3	53.5	24.6
			25.5	24.5	25.2	27.0
			5.8	5.5	4.4	3.8
			9.7	10.3	9.4	8.5
			16.3	15.6	16.3	23.3
			125.4	131.2	108.8	87.2
			NAPHTHALENE ACETIC ACID			
			46.8	44.6	40.6	0.87
			22.5	22.9	24.4	23.5
Hypocotyl..... First internode..... Roots.....  Total.....	11.2 7.1 12.0  101.7	12.2 7.5 18.0  122.2	11.0	10.7	11.8	9.3
			7.0	5.5	5.0	3.1
			11.6	12.9	16.3	12.7
			98.9	96.6	98.1	49.5

#### TUMOR FORMATION, BUD INHIBITION, AND MOBILIZATION

It has been found (5) that secondary thickening, accompanied by relatively slight cellular proliferation and root initiation, was induced in stems of bean plants within a period of approximately 20 days following treatment with naphthalene acetamide. HAMNER and KRAUS (2) reported that treatment of bean plants with naphthalene acetic and indoleacetic acids resulted in marked cellular proliferation, tumor formation, and the initiation of numerous root primordia

within 96 hours after treatment. Experiments were conducted to compare these, and additional responses resulting from use of the acetamide, with those observed when the plants were treated with naphthalene acetic and indoleacetic acids.

Approximately 500 uniform plants were divided into four equal groups and the second internodes severed 1.5 cm. above the node to which the primary leaves were attached. Plants of each group were treated by applying approximately 2 cu. mm. of 2 per cent lanolin mixtures of naphthalene acetamide, naphthalene acetic acid, and

TABLE 3

EFFECT OF TERMINAL APPLICATIONS OF 2 PER CENT MIXTURES OF LANOLIN AND NAPHTHALENE ACETAMIDE, INDOLEACETIC ACID, AND NAPHTHALENE ACETIC ACID TO DECAPITATED BEAN STEMS. FIGURES REPRESENT GRAMS DRY WEIGHT PER 100 PLANTS

TREATMENT	WET WEIGHT		DRY WEIGHT		LENGTH OF AXILLARY SHOOTS (CM.)
	AXILLARY SHOOTS	TREATED TIP*	AXILLARY SHOOTS	TREATED TIP*	
Control. ....	307.1	11.2	28.3	1.2	4.96
Naphthalene acetamide..	191.5	25.5	19.9	3.2	1.79
Indoleacetic acid. ....	186.2	44.8	18.3	5.2	2.37
Naphthalene acetic acid..	131.1	73.1	12.7	9.4	1.56

\* Portion of first internode treated following decapitation.

indoleacetic acid to the freshly cut surfaces. Those of the fourth group were treated with pure lanolin as controls.

Measurements made 7 days after treatment showed that all three compounds inhibited growth of axillary shoots and induced mobilization of solid matter in the treated first internodes, as evidenced by the dry weights of these portions (table 3). Plants treated with acetamide showed no external evidence of a tumor near the region to which the chemical was applied (fig. 6), but this portion (first internode) weighed approximately 166 per cent more than comparable portions of control plants at the end of the experiment. Transverse sections made near the region treated with the acetamide showed much more secondary thickening than comparable sections from controls, the new cells arising mainly from the cambium without initiation of root primordia. In contrast to this, marked cellular

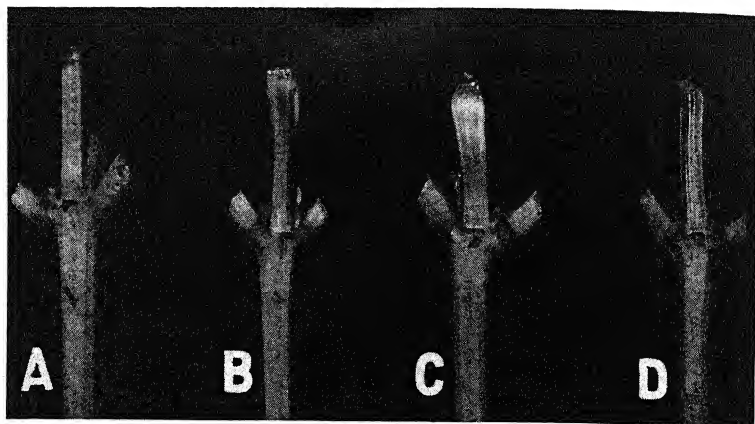


FIG. 6.—Effect of terminal applications of *A*, pure lanolin; *B*, 2 per cent mixtures of lanolin-indoleacetic acid; *C*, lanolin-naphthalene acetic acid; and *D*, lanolin-naphthalene acetamide. Five days after application naphthalene acetamide failed to induce tumor development.

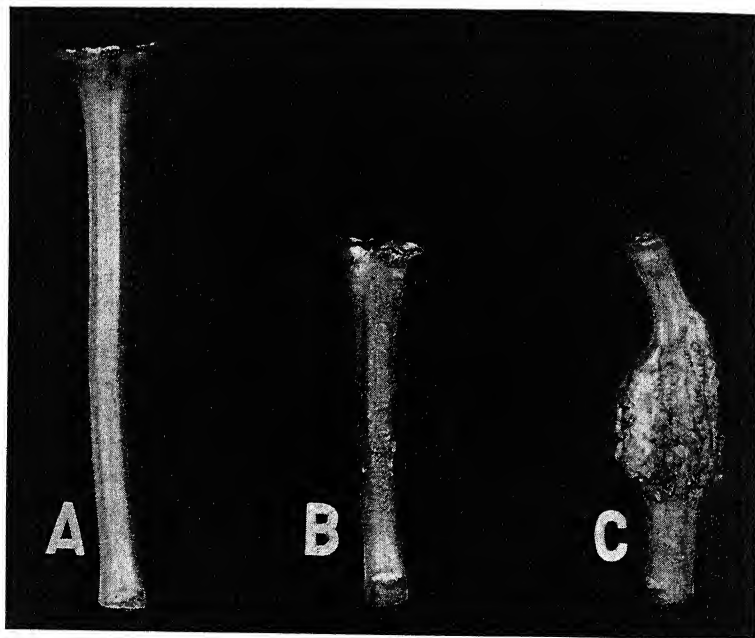


FIG. 7.—Response of first internodes of bean plants 2 weeks after treatment with: *A*, pure lanolin; *B*, 2 per cent naphthalene acetamide-lanolin paste; *C*, 2 per cent naphthalene acetic acid paste, applied as band around center of internode.

proliferation of several tissues followed application of naphthalene acetic acid and resulted in the formation of roots within 7 days fol-



FIG. 8.—Effect of ringing hypocotyl of bean cutting with 2 per cent lanolin-naphthalene acetamide mixture (left) compared with untreated cutting. Photographed 2 weeks after treatment.

lowing treatment (2). Thus mobilization of materials was associated mainly with secondary thickening in plants treated with the aceta-

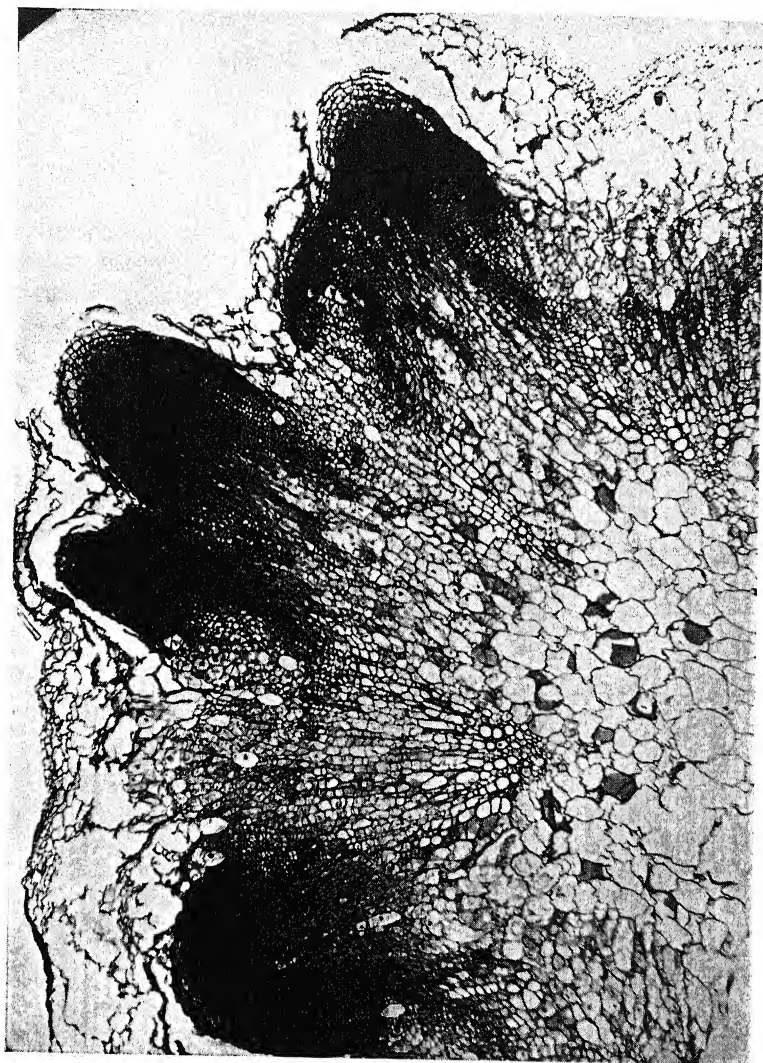


FIG. 9.—Transverse median section of tumor resulting from application (narrow ring) of 2 per cent naphthalene acetic acid-lanolin mixture 96 hours after treatment. Cells of cortex have enlarged; endodermal cells proliferated. Root primordia derived mainly from ray cells have ruptured the cortex. Pith shows little or no response. Same magnification as fig. 10.

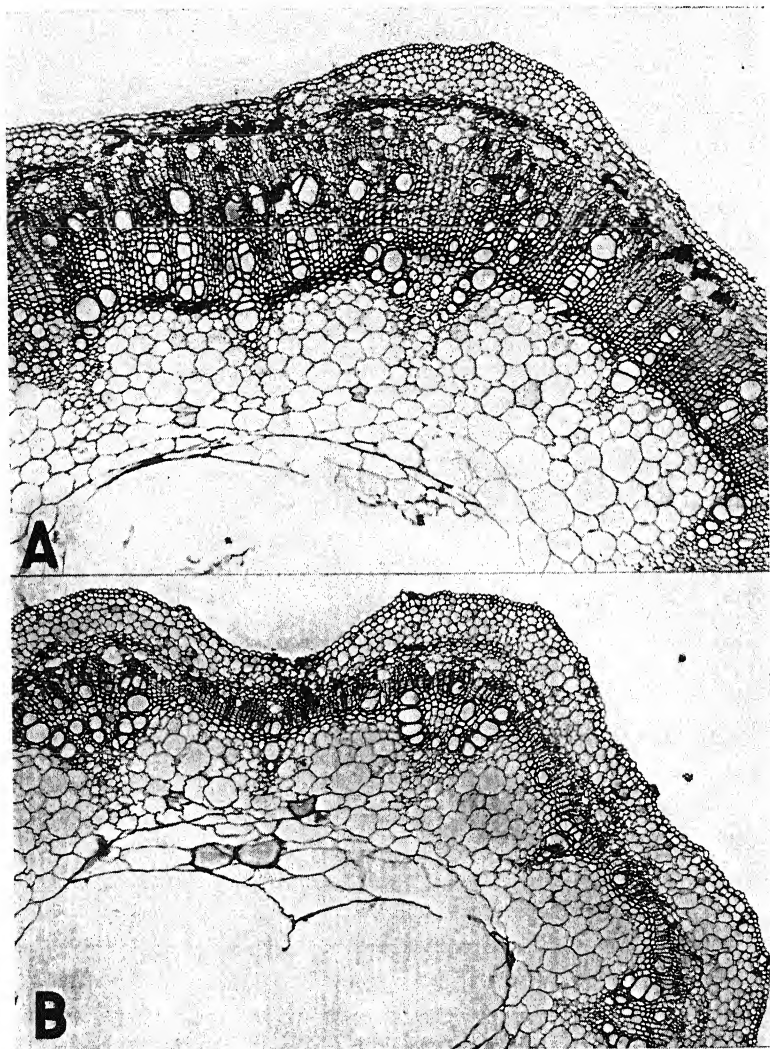


FIG. 10.—*A*, transverse median section through region over which narrow ring of 2 per cent naphthalene acetamide-lanolin mixture had been placed 216 hours after application. *B*, similar section from stem treated with lanolin after same period of time. Most obvious differences are increased thickening of walls of pericyclic fibers, somewhat wider cambial zone, much greater amount of secondary xylem, and thickening of walls of outer ray cells in *A*. Cf. fig. 9; same magnification.

mide (5). Naphthalene acetic acid likewise induced mobilization of materials, but in this instance mobilization was associated with marked cellular proliferation of various tissues of the stem.

Further experiments were conducted in which lanolin containing different concentrations of naphthalene acetamide and naphthalene

TABLE 4

EFFECT OF VARIOUS LANOLIN-NAPHTHALENE ACETIC ACID AND LANOLIN-NAPHTHALENE ACETAMIDE MIXTURES APPLIED AS A RING TO FIRST INTERNODES OF BEAN SEEDLINGS. FIGURES REPRESENT GRAMS DRY WEIGHT OF PARTS FROM 100 PLANTS; AVERAGE LENGTH AND DIAMETER OF 50 PARTS

PLANT PART	PERCENTAGE NAPHTHALENE ACETIC ACID				PERCENTAGE NAPHTHALENE ACETAMIDE				
	2.0	0.02	0.002	0.0002	2.0	0.02	0.002	0.0002	0.0
DRY WEIGHT (GM.)									
Leaves.....	53.9	58.1	58.5	57.7	66.2	58.1	58.3	58.3	61.8
Hypocotyl.....	20.5	19.3	20.5	17.8	17.5	16.7	17.4	17.1	15.3
First internode....	20.2	12.8	11.9	11.4	8.9	11.7	10.2	9.5	9.0
Tip*.....	5.4	9.8	9.1	13.0	7.3	13.5	14.1	15.1	13.8
LENGTH (CM.)									
Petioles.....	3.8	5.7	6.7	6.9	4.3	6.6	6.9	6.9	7.0
Hypocotyl.....	6.5	6.5	6.4	6.2	6.1	6.3	6.4	6.5	6.2
First internode....	3.6	4.0	4.4	5.3	3.7	4.7	5.3	5.2	5.2
Tip*.....	0.5	3.4	4.6	5.9	1.0	6.6	6.8	6.5	5.8
DIAMETER (CM.)									
First internode†...	6.4	4.9	3.7	3.6	3.4	3.4	3.4	3.2	3.4

\* All above second node.

† Across treated region.

acetic acid was applied as a narrow band, 2 mm. wide, around the first internodes of bean plants. The mixtures were prepared as described earlier (6) except that tertiary butyl alcohol was used as a solvent. In these experiments uniform plants grown in soil were used when approximately 10-12 inches high and having first internodes 3-4 inches in length.

Within 2 hours after treatment with the naphthalene acetic mixture, all plants except those treated with the lowest concentration showed curvatures of the stems, while none of those treated with the acetamide curved. As in the case of terminal applications following decapitation, lateral applications of the acid stimulated cellular proliferation of several tissues of the stem and initiation of root primordia (figs. 7, 9; table 4). In contrast to this response, lateral treatments with the acetamide induced more limited cellular proliferation, evidenced as secondary thickening (fig. 10). Root primordia were not initiated in the stems nor was there a significant increase in the diameter of the treated section. Both compounds inhibited development of the terminal bud (table 4).

Two per cent lanolin-naphthalene acetamide mixture greatly inhibited expansion of the terminal buds when applied in the form of a band on the hypocotyl of bean cuttings (fig. 8). Two per cent lanolin mixtures of naphthalene acetamide placed on the cut surfaces of decapitated stems of soybeans and etiolated pea seedlings likewise inhibited growth of the lateral buds.

### Conclusions

The results of these experiments indicate that marked cellular proliferation was closely associated with mobilization of solid materials toward that part of the stem to which naphthalene acetic acid was applied. On the other hand, application of naphthalene acetamide also induced mobilization of solid materials, but in this case it was accompanied (at least during the interval immediately following treatment) by very little cellular proliferation and no appreciable increase in the diameter of the treated part. Although the amount of cellular proliferation induced by these two compounds was clearly different, both greatly inhibited growth of terminal and lateral buds.

The data show that most of the responses to both compounds were similar in nature, but responses following treatment with acetamide occurred more slowly than those with the acid. Responses common to both compounds were: negative curvatures in stems of etiolated pea, bean, and of *Avena* coleoptiles, and delayed tumor and root formation on beans. Dissimilar responses were also observed, those specifically associated with the acetamide being: (1) lignification of

bean stems associated with increased cambial activity, a response never obtained with application of very small amounts of naphthalene acetic acid; (2) increased and more fibrous root growth of bean plants sprayed with the acetamide; and (3) leaf curling following application of lateral treatments to stems of petunia.

### Summary

1. A comparison was made of some of the responses of certain plants when treated with naphthalene acetamide and naphthalene acetic acid.

2. Naphthalene acetamide caused growth curvatures when used in the pea test, and in *Avena* coleoptiles when applied unilaterally in the form of a 0.02 per cent lanolin mixture. When applied to coleoptiles in relatively low concentrations (250 gamma per liter), using the usual *Avena* test with agar blocks, the acetamide failed to give growth curvatures. Naphthalene acetic acid caused growth curvatures in both the pea and *Avena* test at concentrations of 1 mg. per liter and 250 gamma per liter, respectively.

3. Unilateral applications of lanolin containing naphthalene acetamide (2 per cent) on stems of etiolated pea and kidney bean plants resulted in negative curvatures. No apparent curvatures resulted when the stems of four o'clock, soybean, and tomato plants grown in natural light were treated unilaterally with acetamide. Petunia and kidney bean plants treated in this manner showed slight negative curvatures. Applications of a 2 per cent mixture of lanolin and naphthalene acetic acid resulted in first negative then positive bending in all except kidney bean plants grown in light. Negative curvatures were interpreted as resulting from initially low concentrations of the acid. Growth was apparently inhibited, on the side of the stem nearest the application, as the concentration of acid in the tissues became greater.

4. Naphthalene acetamide and naphthalene acetic acid were applied to bean plants in the form of emulsion sprays. Both compounds inhibited development of the terminal buds and expansion of primary leaves when used in strong concentration. The acetamide stimulated root growth over that of untreated plants while the acid was ineffective in this respect. The less concentrated acetamide

sprays accelerated top growth to some extent while the acid sprays had no noticeable effect.

5. Terminal applications of 2 per cent lanolin-naphthalene acetamide or naphthalene acetic acid mixture to decapitated beans or lateral applications on the stems resulted in mobilization of solid materials toward the treated region. Mobilization was associated with appreciable secondary thickening in plants treated with naphthalene acetamide, and with marked cellular proliferation when naphthalene acetic acid was applied.

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# GROWTH SUBSTANCES AND GAMETIC REPRODUCTION BY PHYCOMYCES<sup>1</sup>

WILLIAM J. ROBBINS

(WITH FIVE FIGURES)

## Introduction

Although numerous observations (3) on the formation of zygotes by the Mucoraceae were made by the TULASNE brothers, DE BARY, BREFELD, VAN TIEGHEM, LE MONNIER, BAINIER, THAXTER, and others after the first observation by EHRENBERG on *Sporodinia grandis* in 1820, a correct understanding of the process dates from the studies of BLAKESLEE (1). BLAKESLEE's discovery that *Phycomyces* is heterothallic made possible an attack on the physiology of gametic reproduction of that organism, and several investigators have made contributions to the problem.

ORBAN (5) reported that 1 per cent  $MnCl_2$  added to a 3 per cent biomalt and 2 per cent agar medium permitted vigorous vegetative growth but inhibited gametic reproduction. In fact, additions of  $NaCl$ ,  $Ca(NO_3)_2$ , and other salts decreased zygote formation, caused the development of various abnormalities, and at higher concentrations inhibited gametic reproduction.

SWARTZ (17) and RONSDORF (7) found that the most favorable temperature for zygote production was 20° C. or somewhat lower, and in their experiments at temperatures above 22° gametic reproduction was reduced or entirely inhibited.

BURGEFF (2) presented evidence that substances from the plus strain diffusible through a celloidon membrane were responsible for the initial development of zygophores of the minus strain, and vice versa. RONSDORF (7) found that the larger amounts of nitrogen inhibited formation of zygotes, in agreement with the observations by BENECKE, KLEBS, and others for other plants.

Study of the nutritive aspects of gametic reproduction by

<sup>1</sup> Assistance in this work was furnished by the personnel of Works Progress Administration Official Project 465-97-3-70. Special thanks are due V. N. SERGIEVSKY.

*Phycomyces* was handicapped, however, because growth of the organism could be secured only on media to which some supplement of natural origin was added; for example, an agar containing 3 per cent malt was frequently used. Supplements of natural origin are variable in character and complex in makeup, and their effects are therefore difficult to evaluate.

In a series of papers published from 1931 to 1934, SCHOPFER reported observations on the formation of zygotes by *Phycomyces blakesleeianus*. He observed that little growth developed and few or no zygotes were produced on an agar containing pure sugar, asparagine, and mineral salts. Additions to this medium of dried brewers' yeast, extracts of yeast, vitamin B concentrate, malt extract, extracts of rice polishings, wheat germ (10, 13, 15), or the substitution for the pure sugar of impure maltose or impure sucrose (12) caused material increase in growth and the formation of 1000 or more zygotes in a single petri dish. SCHOPFER (8, 9) concluded that vitamin-like substances are concerned in the growth and in the gametic reproduction of *Phycomyces*.

In 1934 SCHOPFER (11, 14) reported that the addition of crystalline thiamin (vitamin B<sub>1</sub>) to an agar medium containing pure sugar, asparagine, and mineral salts permitted growth of *Phycomyces* and induced the development of zygotes. His discovery that *Phycomyces* would grow vigorously in a solution of sugar, asparagine, mineral salts, and thiamin and the availability of all these substances in chemically pure form permit further investigation of the nutritive aspects of gametic reproduction by this fungus.

Although SCHOPFER was not certain that thiamin was the sole effective agent in the preparations of natural origin which he had found beneficial for *Phycomyces*, the conclusion to be drawn from his work is that a medium of sugar, asparagine, mineral salts, and thiamin is adequate for its growth and gametic reproduction.<sup>2</sup> Several investigators (including the writer) as well as later work by SCHOPFER have amply confirmed his original findings on the adequacy of such a medium for the vegetative growth of *Phycomyces*;

<sup>2</sup> SCHOPFER (14) gives the following as a synthetic medium necessary for *Phycomyces*: glucose 10 per cent; asparagine 0.2 per cent; MgSO<sub>4</sub>·7H<sub>2</sub>O 0.05 per cent; KH<sub>2</sub>PO<sub>4</sub> 0.15 per cent; crystalline thiamin (vitamin B<sub>1</sub>) 25 gamma per cent.

and at temperatures of 20° or somewhat lower gametic reproduction occurs on the same medium. At 25° C., on a medium supplemented with thiamin alone, gametic reproduction is absent or scanty, and experiments at that temperature reported in this paper suggest that growth substances in addition to thiamin are concerned in the gametic reproduction of *Phycomyces*.

### Material and methods

The plus and minus strains of *Phycomyces blakesleeanus* were originally secured from Dr. A. F. BLAKESLEE. Two basic culture solutions were used, each of which was supplemented with 0.01 ppm boron, 0.01 ppm molybdenum, 0.20 ppm iron, 0.04 ppm copper, 0.15 ppm zinc, and 0.02 ppm manganese. Solution 1 contained per liter 50 gm. dextrose, 0.5 gm. asparagine, 0.5 gm.  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 1.5 gm.  $\text{KH}_2\text{PO}_4$ , and 0.25 mg. thiamin. Solution 2 was solution 1 with 100 gm. dextrose and 1 gm. asparagine. The asparagine was purified by recrystallization from alcohol; the thiamin was Merck's synthetic; the other chemicals were of chemically pure grade. Difco standardized agar was used. Petri dishes 10 cm. in diameter and 1.5 cm. deep were used as culture vessels and 25 ml. of medium was placed in each dish. Sterile conditions were maintained throughout; the media were sterilized in an autoclave at 15 lb. pressure for 20 minutes. Drops of a spore suspension of *Phycomyces* in sterile distilled water were used as inoculum and the cultures were incubated at 25° C., except where otherwise noted, in weak diffuse daylight. The formation of progametes and zygotes was observed under a hand lens or a binocular microscope. Only those zygotes were recorded which showed black appendages partially or completely developed; no attempt was made to distinguish separately those which were fully matured.

### Experimentation

Interest in this problem had been aroused by difficulties experienced in obtaining zygotes of *Phycomyces* with media upon which they were expected to develop. It was assumed that a solution of sugar, asparagine, mineral salts, and thiamin would be satisfactory, but repeated experiments with such medium solidified with agar

gave negative or unsatisfactory results under the conditions employed.

Growth on media to which no thiamin was added was extremely scanty; few or no sporangiophores were produced and no progametes or zygotes developed. When thiamin was added growth was profuse, aerial mycelium developed, abundant sporangiophores and sporangia were produced, but few or no progametes formed. The production of progametes and zygotes was materially increased, however, if in

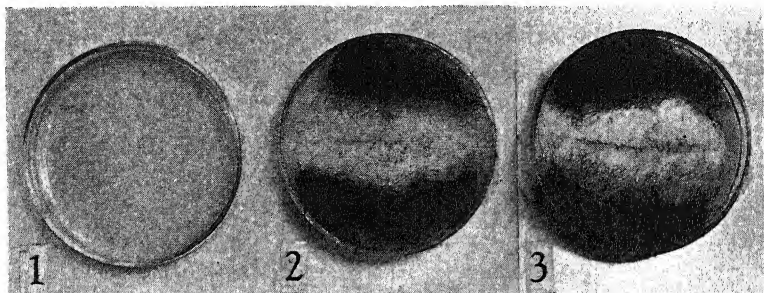


FIG. 1.—Effect of thiamin and oatmeal extract on gametic reproduction. All petri dishes contain same amounts of dextrose, asparagine, and mineral salts and 1 per cent agar. 1, no thiamin; 2, 0.00625 mg. thiamin; 3, 0.00625 mg. thiamin and extract of 0.625 gm. oatmeal. Age 11 days.

addition to thiamin an extract of some material of natural origin was included with the agar, sugar, asparagine, and mineral salts.

This is illustrated in figure 1. In this instance solution 2 with 2 per cent agar was used. The medium in each dish was inoculated at opposite edges with the plus and minus strains and the cultures were photographed after 11 days of incubation. Growth on agar containing dextrose, asparagine, and mineral salts but no thiamin was too sparse to show in the photograph. On the same medium plus thiamin the growth was profuse and abundant sporangia were formed. The line of progametes was too thin to be visible in the photograph, however, and a total of seven zygotes with black appendages partly or completely developed was formed on four dishes—fewer than two per plate. The addition of a water extract from 0.625 gm. of oatmeal<sup>3</sup> per plate to the medium containing thiamin resulted in a

<sup>3</sup> The dry matter in the extract added to each petri dish was 14 mg.

heavy line of yellow progametes and increased the number of zygotes nine times.

Other experiments carried out in the same way with a 1 per cent agar containing dextrose, asparagine, mineral salts, and thiamin gave entirely negative results, so far as progametes and zygotes were concerned, when no extract was added to the medium.

A comparison of these experiments with those of SCHOPFER (14) revealed certain differences. In addition to the difference in strains of the fungus employed and in the source of the chemicals, SCHOPFER used 3 per cent agar instead of the 1 or 2 per cent in my media; his temperature of incubation was 18° to 20° C. and mine 25° C.; he inoculated the petri dishes in six places arranged in two parallel lines about 3 cm. apart, as judged from the photographs reproduced in his paper. Although some of these differences were originally considered insignificant, it seemed advisable to determine their importance as well as that of certain other factors.

#### METHOD OF INOCULATION

In the original experiments the agar medium in the petri dishes was inoculated at one edge with a drop of a spore suspension of the plus strain of *Phycomyces*, and at the opposite edge with the minus strain. The distance between the two drops was about 8.5 cm. SCHOPFER used six drops and the distance between the inocula of the plus and minus strains appears to have been about 3 cm. Furthermore, in one or two instances in my first experiments the dishes were tilted in handling and the drops of inoculum ran over the surface of the agar, bringing the mycelia close together from the beginning of the growth period. Under such circumstances progametes were formed on the same medium on which none developed when the inocula remained in their original locations near opposite edges of the dish.

Because of this observation the effect of the method of inoculation was investigated. Three drops of a spore suspension of the plus strain were equally spaced in a line across the dish, and three drops of the minus strain were arranged in a line parallel with the first and 2.5-3 cm. away, with the result that the initial mycelia of the plus and the minus strains were 2.5-3 cm. apart instead of 8.5 cm. as in

the first method of inoculation described. Because of the shorter distance separating them the two mycelia met earlier in their growth. With this method of inoculation progametes and zygotes were produced regularly on agar media on which the other method of inocula-

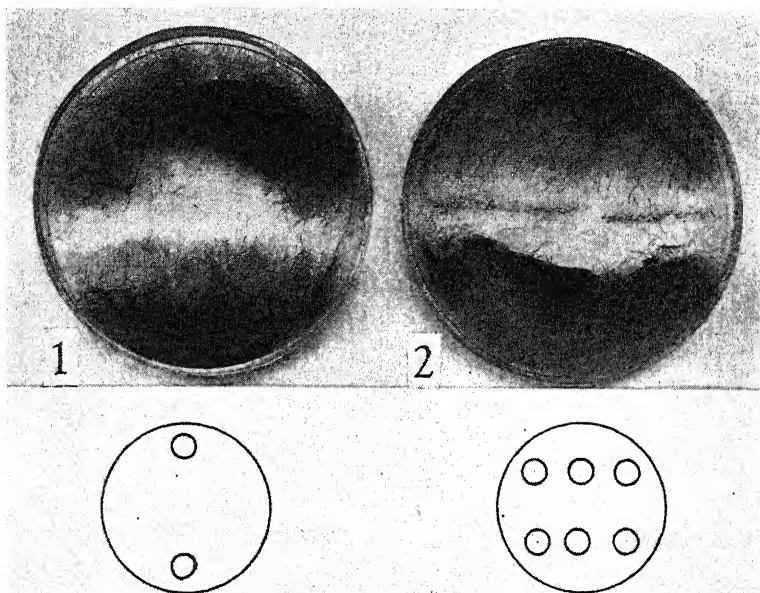


FIG. 2.—Effect of method of inoculation on gametic reproduction. Both dishes contain 1 per cent agar with same amount of dextrose, asparagine, mineral salts, and thiamin. 1, inoculated at opposite edges of dish; 2, inoculated in six places arranged in two lines about 3 cm. apart. Sketches show method of inoculation. Age 11 days.

tion gave little or no result (fig. 2). In this experiment the medium consisted of solution 2 containing 1 per cent agar.

It might be suggested that the distance separating the inocula was not the factor responsible for the difference in gametic reproduction noted, but that the results were related to the quantity of inoculum. In the first method only one drop each of a spore suspension of the plus and minus strains was used while in the second three drops of each were used in each plate.

That distance between the inocula and not quantity of inoculum

was the important factor was demonstrated by placing a drop of a spore suspension of each of the two strains at different distances from one another on media in petri dishes.

In one experiment solution 1 containing 1 per cent Difco agar was prepared and poured into petri dishes. Quadruplicate plates were inoculated with one drop of a spore suspension of each strain, the drops being placed 1, 2, 3, 5, 6, and 8 cm. apart. In addition, in one set of plates three drops of each strain were arranged in parallel lines

TABLE 1

DISTANCE SEPARATING INOCULA AND DEVELOPMENT OF PHYCOMYCES. ALL GROWN ON SAME 1 PER CENT AGAR MEDIUM CONTAINING DEXTROSE, ASPARAGINE, MINERAL SALTS, AND THIAMIN (FIG. 3)

DISTANCE SEPARATING INOCULA (CM.)	MYCELIA MET AFTER	PROGAMETES OBSERVED AFTER	LENGTH OF LINE OF PROGAMETES (CM.)	YOUNG SPORANGIO-PHORES FORMED AFTER	NO. ZYGOTES PER PLATE
1.0.....	30 hours	66 hours	5.0-5.5	66 hours	9
2.0.....	49	66	4.0-6.0	66	15
3.0.....	62	98	4.5	66	2
5.0.....	66	100	3.0	66	1
6.0*	98	None	.....	66	0
8.0.....	98	None	.....	66	0
3.0†	66	68	8.5	66	4

\* One culture in four showed a few progametes.

† Inoculated in six places in two parallel lines about 3 cm. apart.

about 3 cm. apart. Observations were made at intervals in order to fix as nearly as practical the time when the mycelia of opposite strains met, the time progametes were observed, and the time young sporangiophores appeared. After 10 days the length of the line of progametes was measured and the number of zygotes with appendages was counted (table 1; fig. 3).

No progametes were produced in the plates when the original inocula were 8 cm. apart and only one plate in four showed a few when the separating distance was 6 cm. When the distance between the original inocula was 1, 2, 3, or 5 cm., lines of progametes were produced; but at the latter two distances the line was short. The superiority of the shorter distances between the inocula was evident also in the number of zygotes with appendages. When the inocula

were 1-2 cm. apart, nine or fifteen zygotes per plate appeared; for greater distances the number was reduced to two, one, or none (table 1).

Why should the distance between inocula make a difference in the gametic reproduction of *Phycomyces*? An unqualified answer cannot

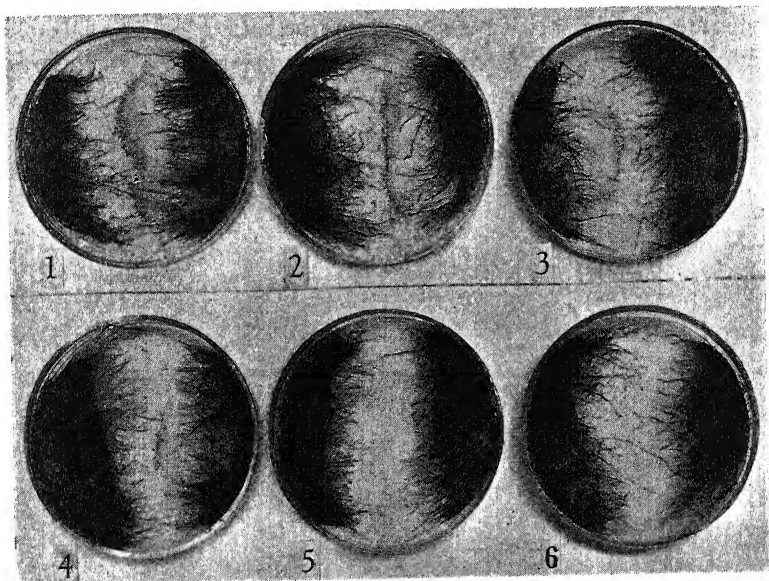


FIG. 3.—Distance between inocula and gametic reproduction. All dishes contain 1 per cent agar with same amounts of dextrose, asparagine, mineral salts, and thiamin and were inoculated with a drop of spore suspension of plus and minus strains. 1, distance between inocula 1 cm.; 2, distance 2 cm.; 3, 3 cm.; 4, 5 cm.; 5, 6 cm.; 6, 8 cm. Age 9 days.

be given to this question. There seemed to be some relation between the age of the mycelium and the development of the progametes. If none was formed within 3 or 4 days from the time of inoculation, none developed later. In fact, unless the opposing mycelia grew together within 3 days from the time of inoculation, no progametes were produced under the conditions of these experiments. Formation of sporangiophores and sporangia seemed to be associated in some way with inhibition of the production of progametes. If sporangiophores and sporangia developed freely before the two op-

posite mycelia grew together, formation of progametes was likely to be inhibited. It appeared, furthermore, that once production of sporangia was well under way the ripening and maturation of the zygotes stopped.

This apparent antagonism between the formation of sporangiophores and gametic reproduction in *Phycomyces* would be understandable on the assumption that growth substances other than thiamin are concerned in the development of this organism, and that these substances are exhausted by the formation of sporangiophores and sporangia, either by being used in the formation of these agametic structures or by being destroyed by autolysis (which probably begins with the production of sporangia).

#### CONCENTRATION OF AGAR

SCHOPFER (14) used a 3 per cent agar in his experiments on the effect of crystalline thiamin on gametic reproduction by *Phycomyces*.

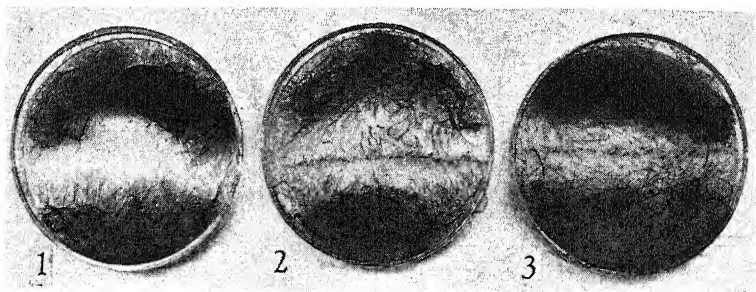


FIG. 4.—Effect of concentration of agar and of potato extract on gametic reproduction. All dishes contain same amounts of dextrose, asparagine, mineral salts, and thiamin and were inoculated at opposite edges with plus and minus *Phycomyces*. 1, contains 1 per cent agar; 2, 3 per cent; 3, 1 per cent agar and per plate the extract of 11 gm. of potato. Age 11 days.

It appeared that the concentration of agar was of importance in my experiments; more profuse gametic reproduction was secured on the higher concentrations. When the distance between inocula of the two strains was 8–8.5 cm., progametes were rarely secured at 25° C. on a 1 per cent agar made up with solution 1 or 2. On a 2 per cent agar progametes were formed but few zygotes developed; with 3 per cent the development of both was more profuse.

The effect of agar concentration is shown in figure 4. In this in-

stance solution 2 was used. The dishes were inoculated with the plus and minus strains at opposite edges and photographed after 11 days. On the 1 per cent agar no progametes were formed although the vegetative growth was good. On the 3 per cent agar a line of progametes developed and zygotes were produced. It is of interest to note that the addition to the 1 per cent agar medium of the extract of 2.75 gm. of potato<sup>4</sup> per plate gave results similar to those found on the 3 per cent agar to which no such natural supplement

TABLE 2  
EFFECT OF CONCENTRATION OF AGAR ON GAMETIC  
REPRODUCTION BY PHYCOMYCES

MEDIUM	TWO INOCULATIONS 8.5 CM. APART		SIX INOCULATIONS IN TWO LINES 3 CM. APART	
	PROGAMETE PRODUCTION	NO. ZYGOTES PER PLATE	PROGAMETE PRODUCTION	NO. ZYGOTES PER PLATE
Solution 2 plus 1% agar....	None	None	Line 2 mm. wide	3
Solution 2 plus 3% agar....	Line 2 mm. wide	31	Line 3 mm. wide	56
Solution 2 plus 1% agar....	.....	.....	Line 2 mm. wide	4
Solution 2 plus 3% agar....	.....	.....	Line 3 mm. wide	46

was added; in other words, the higher concentration of agar without potato extract had much the same effect on gametic reproduction as the addition of potato extract to the lower concentration.

Table 2 summarizes the results of experiments in which the effect of 1 per cent and 3 per cent agar was compared. The superiority of a medium containing 3 per cent agar over the same medium containing 1 per cent is shown. The favorable effect of the higher concentration was evident both when two inoculations at opposite edges of the dish were used and when six inoculations in two lines about 3 cm. apart were employed; although in every instance superior results were obtained with the latter method. SCHOPFER (9) also found the higher concentrations of agar favorably to affect gametic reproduction by

<sup>4</sup> The dry matter in the extract added to each petri dish was 44 mg.

*Phycomyces*. He considered, however, that the agar did not exert a chemical action, but that its effect was physico-chemical because of its density, cohesion, and osmotic pressure resulting from its colloidal character. Apparently agar contains substances which beneficially affect gametic reproduction; otherwise it would seem difficult to explain the similarity of the results obtained by increasing the concentration of agar and those secured when potato extract was added to the more dilute agar. Furthermore, the beneficial material can be extracted in part from the agar.

#### EFFECT OF PURIFYING AGAR

If the assumption is correct that agar contains some substance or substances important in gametic reproduction, then it should be possible to remove these substances and reduce the effectiveness of the agar. An attempt to purify agar by extraction with methyl alcohol, or by aqueous pyridine, was partially successful.

Difco agar was dissolved in water by heating and the hot liquid poured with stirring into redistilled methyl alcohol. The precipitate was centrifuged off, redissolved in hot water and reprecipitated with alcohol, removed by centrifuging, washed with 60 per cent methyl alcohol, and dried. The methyl alcohol extracts were combined and evaporated by boiling to small volume. About half the agar remained in the extract.

Solution 2 was prepared with 1 per cent Difco agar, 1 per cent Difco agar purified with methyl alcohol, and with the methyl alcohol extract sufficient to make a 1 per cent agar.

The plates (in quadruplicate) were inoculated in six places with the two strains of *Phycomyces*. On the Difco agar a line of progametes was produced and six zygotes per plate developed (table 3). On the purified agar the line of progametes was thinner and the zygotes fewer. The line of progametes on the medium prepared with the extract was intermediate in thickness between these two and the number of zygotes was no greater than on the purified agar. This indicated that there were materials in Difco agar beneficial to gametic reproduction and that they could be extracted in part with methyl alcohol.

Dry powdered Difco agar was extracted at room temperature over

a period of 48 hours with three lots of 5 per cent aqueous pyridine. The extracts were combined and evaporated to small volume. The agar was washed with 95 per cent alcohol and dried. Most of the agar was recovered.

Solution 1 was prepared with 1 per cent Difco agar, 1 per cent agar purified with pyridine, 3 per cent agar purified with pyridine, and 1 per cent purified agar plus the extract equivalent to 1 per cent agar

TABLE 3

EFFECT ON GAMETIC REPRODUCTION OF PURIFYING AGAR WITH METHYL ALCOHOL OR AQUEOUS PYRIDINE. ALL MEDIA CONTAINED DEXTROSE, ASPARAGINE, MINERAL SALTS, AND THIAMIN

MEDIUM	SIX INOCULATIONS		TWO INOCULATIONS	
	PROGAMETE PRODUCTION	NO. ZYGOTES PER PLATE	PROGAMETE PRODUCTION	NO. ZYGOTES PER PLATE
Solution 2 plus				
1% Difco agar.....	Line 2 mm. wide	6	.....	.....
1% agar purified with methyl alcohol.....	Thin line	2	.....	.....
methyl alcohol extract equal to 1% agar.....	Thin line+	1	.....	.....
Solution 1 plus				
1% Difco agar.....	Line 2 mm. wide	4	None	None
1% agar purified with pyridine.....	Very few	None	None	None
3% agar purified with pyridine.....	Line 2 mm. wide	15	Thin line	3
1% purified agar extract equivalent to 1% agar....	Line 2 mm. wide	3	None	None

(fig. 5). Plates in duplicate were inoculated at opposite edges with a drop of a spore suspension of the two strains of *Phycomyces*; plates in duplicate were also inoculated with six drops of spore suspension arranged in two lines about 3 cm. apart. The progamete and zygote production was better on the 1 per cent Difco agar than on the 1 per cent purified agar (table 3). Addition of the extract to the purified agar improved the medium but did not make it superior to the 1 per cent unpurified agar. The results on the 3 per cent purified agar were superior to those on the 1 per cent unpurified agar, but not equal to those secured earlier on a 3 per cent unpurified agar.

It appeared, therefore, that the agar contained material favorable to gametic reproduction by *Phycomyces*, and that this material could be extracted in part by dilute methyl alcohol or by aqueous pyridine.

What is this beneficial material? It seems to be organic rather than inorganic, and probably belongs in the class of growth substances (6). Extraction of the agar with pyridine reduced the ash from 3.56 to 2.41 per cent. Addition of agar ash, however, prepared by ashing at low red heat and then heating to dryness with HCl, did not improve growth or gametic reproduction on the purified agar, and was ineffective when added to a 1 per cent agar medium containing

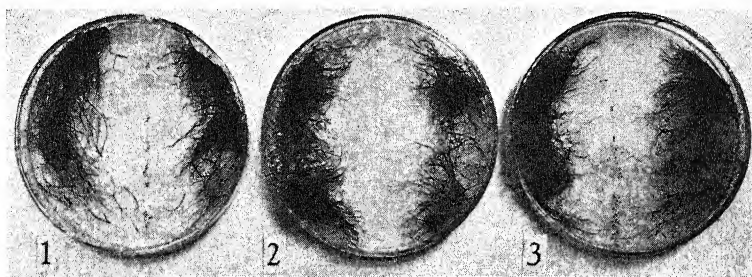


FIG. 5.—Purification of agar and gametic reproduction. All dishes contain 1 per cent agar and same amounts of dextrose, asparagine, mineral salts, and thiamin. 1, Difco agar; 2, same extracted with pyridine; 3, same extracted with pyridine plus extract equivalent to 1 per cent agar. Inoculated in six places. Age 7 days.

thiamin. Increasing the trace elements two and a half, five, or ten times and additions of sufficient zinc to make the total concentration 0.38, 0.75, or 1.5 ppm instead of the 0.15 ppm in the solutions used in this investigation was ineffective. Furthermore, the  $\text{KH}_2\text{PO}_4$  and  $\text{MgSO}_4$  employed in making the media were not specially purified and probably contained many trace elements.

#### EFFECT OF DEXTROSE AND ASPARAGINE

RONSDORF (7) and SCHOPFER (9) found that increased amounts of available nitrogen decreased gametic reproduction of *Phycomyces*. In these experiments also it was observed that the larger amounts of asparagine decreased the number of progametes and zygotes, and sufficiently large amounts (5 or 10 gm. per liter) inhibited them entirely. It appeared that gametic reproduction was interfered with

when either dextrose or asparagine was markedly deficient or was present in large amounts; the asparagine was more effective in this respect than the carbohydrate. From 0.5 to 1 gm. of asparagine per liter and from 10 to 75 gm. of dextrose per liter seemed most favorable.

TABLE 4

AMOUNTS OF ASPARAGINE AND DEXTROSE AND GAMETIC REPRODUCTION OF PHYCOMYCES GROWN ON 1 PER CENT DIFCO AGAR CONTAINING MINERAL SALTS, THIAMIN, AND AMOUNTS PER LITER OF DEXTROSE AND ASPARAGINE GIVEN IN THE TABLE

GRAMS PER LITER		RELATIVE GROWTH	TWO INOCULATIONS		SIX INOCULATIONS	
DEX-TROSE	ASPARAGINE		PROGAMETE PRODUCTION	No. ZYGOTES PER PLATE	PROGAMETE PRODUCTION	No. ZYGOTES PER PLATE
50	0.0	Extremely sparse	None	0	Few	4
50	0.1	Very sparse	Scattered line	2	Few	2
50	0.2	Sparse	None	0	Scattered line	1
50	0.5	Medium	None	0	Line 2 mm. wide	4
50	1.0	Medium+	None	0	Line 2-3 mm. wide	26
50	2.0	Heavy	None	0	Line 4 mm. wide	2
1	0.5	Sparse	None	0	None	0
10	0.5	Medium—	Few	0	Line 2 mm. wide	13
25	0.5	Medium	Scattered line	10	Line 2 mm. wide	31
75	0.5	Medium	Few	0	Line 2 mm. wide	10
100	0.5	Medium	Few	0	Line 2 mm. wide	4
50*	0.5	Medium+	Line 3 mm. wide	43	Line 4 mm. wide	63
50†	0.5	Heavy	Line 4 mm. wide	31	Line 6 mm. wide	24

\* Potato extract equivalent to 2.75 gm. potato per plate added.

† Equivalent to 13.75 gm. of same.

Because of certain other observations of interest, an experiment in which different amounts of dextrose and asparagine were used will be described in detail.

To a medium containing per liter 10 gm. Difco agar, 0.5 gm.  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 1.5 gm.  $\text{KH}_2\text{PO}_4$ , 0.25 mg. thiamin, and the mineral supplements described earlier, the quantities of dextrose and asparagine given in table 4 were added. Plates in duplicate were inoculated at the edges with drops of a spore suspension of the two opposite strains of *Phycomyces*, and duplicate plates were inoculated also with three drops of each strain in parallel lines about 3 cm. apart. The observations are summarized in table 4.

On the media containing 50 gm. of dextrose per liter and no asparagine growth was extremely sparse, demonstrating that the nitrogen in the agar was insufficient in amount or unavailable for the development of *Phycomyces*. The addition of 0.1 gm. or 0.2 gm. of asparagine improved the growth although it was still scant. With still further increase in asparagine growth increased, the heaviest occurring on the medium containing 50 gm. dextrose and 2 gm. asparagine per liter.

Gametic reproduction also was affected by the amount of asparagine. When the inocula of the two strains were located 3 cm. apart and six were used on each plate, the number of progametes increased with increasing quantity of nitrogen up to 2 gm. of asparagine per liter. The number of zygotes per plate was greatest on the medium containing 50 gm. dextrose and 1 gm. asparagine. With smaller amounts of asparagine the number of zygotes was considerably decreased. On a percentage basis, however, the proportion of zygotes to progametes was highest on the medium to which no nitrogen was added. Although the line of progametes was widest on the medium containing 2 gm. of asparagine per liter, few zygotes were formed, and other observations indicate that a larger amount of asparagine inhibited the formation of progametes also.

When the plates were inoculated in two places about 8.5 cm. apart, a scattered line of progametes and two zygotes per plate were formed on the medium containing 50 gm. dextrose and 0.1 gm. asparagine per liter; no progametes were produced on the other media containing 50 gm. dextrose and different amounts of asparagine.

With asparagine constant at 0.5 per liter growth was sparse on the medium containing 1 gm. dextrose per liter and increased with increasing dextrose up to the medium containing 25 gm. per liter. Amounts of dextrose beyond this point up to 100 gm. per liter appeared to have little effect upon growth. With six inoculations per plate no progametes were formed on the medium with 1 gm. of dextrose and 0.5 gm. asparagine per liter, while the number formed with other amounts of dextrose was about the same no matter whether the dextrose was 10 or 100 gm. per liter. The maximum number of zygotes appeared on the plates containing 25 gm. of dextrose per liter; with amounts above and below this quantity the number of

zygotes decreased. With two inoculations per plate few or no progametes were formed and no zygotes were produced except on the medium containing 25 gm. dextrose and 0.5 asparagine, on which a scattered line of progametes and ten zygotes per plate developed.

It is clear from these results that the amount of dextrose and the amount of asparagine affected gametic reproduction. When either was markedly deficient or present in large amounts gametic reproduction was interfered with. Some progametes and zygotes were formed on the low nitrogen media where growth was severely limited and were not produced on the low sugar medium where also growth was scanty. Furthermore it appears from these results that increasing the dextrose or the asparagine did not increase the formation of progametes and zygotes to the extent observed with additions of extracts of potato, corn meal, or oatmeal. This suggests that the effect of these extracts was not associated with assimilable carbohydrate or nitrogen compounds.

#### EFFECT OF PLANT EXTRACTS

SCHOPFER (8, 9, 10, 13, 15, 16) found that extracts of various materials of natural origin favored gametic reproduction of *Phycomyces*, but in his experiments the extracts were added to an agar medium which lacked thiamin. In no instance was the effect of an extract upon gametic reproduction determined in the presence of crystalline thiamin. SCHOPFER's results with extracts of rice polishings, vitamin B concentrate, and other materials appear superior to those he reported for an agar medium supplemented with crystalline thiamin, and he states that he is not certain that the results with the materials of natural origin can be accounted for entirely by their thiamin contents. Nevertheless since thiamin, a necessity for the growth of *Phycomyces*, was a constituent of all the extracts he added to an agar medium lacking that growth substance, it is not possible to assert that the effect of the extracts was not because of their thiamin content. It seemed advisable, therefore, to determine whether extracts of materials of natural origin would affect gametic reproduction when an excess of thiamin was separately supplied. In this way the effect of the thiamin in the natural substances would be eliminated.

Extracts of potato, corn meal, and oatmeal were used since these materials are frequently employed in preparing culture media. The extracts were made by boiling the material with water, or steeping it for a day or two in 50 per cent methyl alcohol. The extracts were decanted, centrifuged, filtered, and concentrated by evaporation. The extract from 0.03 to 12.5 gm. of the original material was added to each petri dish. The experiments will not be described in detail, but the results showed that plant extracts benefited gametic reproduction in the presence of excess thiamin (figs. 1, 4). Their effect therefore appeared to be caused by something other than their thiamin content.

The effect of the agar and plant extracts appeared to be additive; that is, an amount of plant extract which was ineffective or had little effect in a 1 per cent agar medium was decidedly beneficial in the same medium containing 2 per cent agar.

The plant extracts overcame to some extent the inhibitory action of excess nitrogen, or—reversing this statement—the beneficial effect of plant extracts was nullified by increasing the amount of asparagine. For example, no gametes were obtained on a 1 per cent agar medium containing dextrose, mineral salts, thiamin, 5 gm. of asparagine per liter, and the extract of 0.625 gm. of oatmeal per petri dish. When the asparagine was reduced to 1 gm. per liter a line of progametes was produced with the addition of the same quantity of oatmeal extract. No progametes were formed when the oatmeal extract was omitted. These results were obtained with inoculations at opposite edges of the dishes.

The beneficial effect of the plant extracts did not appear to be caused by their content of assimilable nitrogen or carbohydrate. This followed from the observation that an increase in the asparagine or dextrose in the media did not benefit gametic reproduction to the same degree as did the plant extracts (table 4).

SCHOPFER (9) found that the inhibitory action of larger quantities of asparagine could be overcome in part by the addition of impurities extracted from a Kahlbaum maltose, and concluded that the active impurity aids the fungus in resisting the inhibition resulting from the larger quantities of asparagine.

## EFFECT OF LIQUID MEDIA

Since agar appeared to contain substances favorable to gametic reproduction of *Phycomyces* at 25° C., an effort was made to find a medium which was free from such beneficial substances. Asbestos cloth heated in a muffle furnace and washed with HCl and distilled water was saturated with solution 1. *Phycomyces* did not grow on this medium. Asbestos cloth covered with a sheet of glass cloth made from glass wool by suitable heat treatment in a muffle furnace was also ineffective as a solid substratum. On a pad of glass wool covered with a sheet of glass cloth and saturated with solution 1 growth was scanty. Since a solid medium of the type desired was not available, a thin layer of liquid medium in a petri dish was used.

Ten or 15 ml. of solution 1 in a petri dish was inoculated with bits of young mycelium of the plus and minus strains of *Phycomyces*; the bits were placed 1-2 cm. apart so that they soon grew together. A few progametes were produced but no zygotes were observed. The same results were secured when the medium in the dishes was inoculated with the spores of the plus and minus strains. These results are in contrast with those on an agar medium made with the same nutrient solution and inoculated with the two strains 1-2 cm. apart. They suggest that *Phycomyces* will not produce progametes or zygotes in any number at 25° C. in a medium of sugar, asparagine, mineral salts, and thiamin. Furthermore, the addition to solution 1 of potato extract induced the formation of hundreds of progametes and zygotes when the liquid medium was in petri dishes and inoculated with bits of young mycelium or spores. Only one nutrient solution was used in these experiments with liquid media. It is possible that with variations in the sugar, nitrogen, and mineral salts, considerable gametic reproduction can be secured at 25° C. in a medium with no supplement other than thiamin, although there is little encouragement for such a possibility from those experiments in which different quantities of asparagine and sugar were used in an agar medium.

## TEMPERATURE

The preceding experiments were carried out at 25° C. This temperature restricts gametic reproduction by *Phycomyces*. Even on a

favorable medium only a small proportion of the progametes develop into zygotes, and many of the zygotes do not mature. On the other hand vegetative growth is vigorous, and it is more convenient to deal with a small number of zygotes than with the many which form at the lower temperatures.

The effect of temperature on gametic reproduction requires further investigation. It is not likely, however, that a lower temperature of incubation will give results which would materially affect the general conclusions to be drawn from these experiments. At 18°–20° C., distance between inocula and the addition of plant extracts to an agar medium were found to affect the production of zygotes in the same direction as at 25° C. At the lower temperature the number of zygotes is much greater.

### Discussion

These results can best be explained by assuming that growth substances in addition to thiamin must be supplied *Phycomyces* if gametic reproduction is to occur to any extent at 25° C., and that these substances have a beneficial effect at lower temperatures also, although less essential there. Other explanations may be offered: (1) that the effective material is inorganic; (2) that the action of the agar and plant extracts is to nullify some injurious action of the medium; and (3) that we are dealing with a general growth effect not assignable to any specific substances. It is recognized that the validity of the assumption that growth substances in addition to thiamin are concerned in gametic reproduction of *Phycomyces* will not be demonstrated conclusively until such substances are actually isolated and their effects determined.

If growth substances are concerned they should not be regarded as reproductive substances in the sense that they are concerned in gametic reproduction only. It seems more likely that they are required for vegetative growth and other phases of development of the fungus also, and are used up more or less in proportion to the extent and rapidity of growth. The unknown substances are synthesized to some extent by the organism, but under some conditions in amounts inadequate for extensive gametic reproduction.

The writer is inclined to assume the activity of growth substances

rather than inorganic substances because the basic media contained numerous trace elements and additions of agar ash, and increased amounts of trace elements were ineffective. On the other hand, all possible elements in various amounts were not investigated. The hypothetical substances would not appear to consist of available nitrogen or carbohydrate because the nitrogenous and carbon compounds in agar are not available to *Phycomyces*. Furthermore, it is possible on such an assumption to explain other results, as follows:

1. Distance between inocula.—The growth substances in the agar are exhausted in the vegetative growth and formation of sporangia if the inocula are sufficiently separated and contact of the two mycelia is delayed.

2. Inhibitory effect of larger amounts of asparagine.—With increased asparagine vegetative growth is increased and the limited amount of growth substances supplied in the agar is exhausted.<sup>5</sup>

3. Correction of inhibitory effect of asparagine by additions of plant extracts.—The plant extracts increase the amount of growth substances available, which are then adequate to meet the needs for the increased vegetative growth and for gametic reproduction.

4. Effect of plant extracts.—The unknown growth substances are present in the plant extracts.

5. Results at 25° C. in a synthetic liquid medium.—The liquid medium lacks the unknown growth substances, which are synthesized by the fungus in amounts adequate for vegetative growth but not, under the conditions employed, in amounts sufficient for gametic reproduction.

6. Effects of temperature.—Either the formation of the hypothetical substances by the fungus is greater, or the requirement is less, at the lower temperatures.

7. If growth substances are concerned they are water-soluble, soluble in dilute alcohol, and thermo-stable.—The results with pure crystalline B<sub>2</sub> (lactoflavine), pantothenic acid, nicotinic amide, synthetic vitamin B<sub>6</sub>, carotene, and  $\alpha$  tocopherol (vitamin E) have thus far been negative. Biotin (4), pantothenic acid, and heteroauxin (16) have been reported ineffective with *Phycomyces*.

<sup>5</sup> Such an explanation would not apply to amounts of asparagine large enough to interfere with growth.

Even if these assumptions are correct, it should be emphasized that the substances dealt with are probably not the only ones concerned with gametic reproduction in *Phycomyces*. In none of the media used have sex organs appeared on one strain in the absence of the other; yet BURGEFF (2) has shown that substances diffusible through a celloidon membrane from one strain induce development of gametophores in the other. It has already been pointed out (6) that growth substances other than thiamin seem to be concerned in the spore germination of this fungus. It would appear that the relation of *Phycomyces* to an external supply of growth substances may be complex and involve more than the single substance, thiamin.

### Summary

At 25° C. the intensity of gametic reproduction of *Phycomyces blakesleeianus* upon an agar medium containing dextrose, asparagine, mineral salts, and thiamin was affected by the distance between the inocula of the plus and minus strains, by the concentration of the agar, by the addition of plant extracts, by the amount of asparagine, and to a less extent by the amount of carbohydrate. Gametic reproduction was decreased with the greater distance between the inocula, and by the larger amounts of asparagine; it was favored by increasing agar concentration and by the addition of plant extracts. The beneficial materials in agar could be extracted in part by dilute methyl alcohol or by aqueous pyridine. The addition of plant extracts overcame in part the inhibitory effect of the larger amounts of asparagine. Little gametic reproduction was obtained at 25° C. in a liquid medium containing dextrose, asparagine, mineral salts, and thiamin unless a plant extract was added. It is concluded that growth substances in addition to thiamin are probably concerned in the gametic reproduction of *Phycomyces*.

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# EVIDENCE FOR AUXIN PRODUCTION IN ISOLATED ROOTS GROWING IN VITRO<sup>1</sup>

J. VAN OVERBEEK

In an earlier paper it was reported that auxin was found in isolated roots that had been subcultured for 3 weeks (11). The question then arose whether auxin is produced in these isolated roots. Experiments designed to answer this question are reported here.

TABLE 1

TOTAL AMOUNT OF AUXIN PER AVERAGE ROOT IN  $10^{-6}$   $\gamma$  INDOLEACETIC ACID EQUIVALENTS, DURING SUBCULTURING.  $P_0$  INDICATES INITIAL ROOT TIP;  $P_1$ ,  $P_2$ , ETC., INDICATE FIRST CULTURE, SECOND WEEK OF CULTURE (FIRST SUBCULTURE), ETC.

August 2, 1938 $P_0$	230.0	Sept. 24, 1938 $P_2$ Tip	48.4
August 9..... $P_1$ Tip	75.3	Sept. 30..... $P_3$ Tip	50.1
Base	39.2	Base	38.1
Total	114.5	Total	88.2
August 16.... $P_2$ Tip	127.2	October 8..... $P_4$ Entire	
Base	53.6	root	36.5
Total	180.8		

Sufficient peas of the variety Perfection were sterilized and germinated (3) so that more than 1000 root tips 3-4 mm. long and each weighing on the average 10 mg. could be excised. About 200 were analyzed for their auxin content in the manner previously described (8, 9, 11). The remaining tips were cultured in petri dishes containing a basic nutrient solution to which vitamin  $B_1$  was added (3). After one week in culture about 400 roots were analyzed for auxin. The total amount of auxin in these roots ( $P_1$ ) was smaller than that of the initial tip ( $P_0$ ), as shown in table 1. Tips 10 mm. long of the remaining  $P_1$  roots were subcultured and maintained for another week. At the end of this week the roots ( $P_2$ ) had a higher auxin

<sup>1</sup> Work carried out with the aid of the Works Progress Administration, Official Project No. 665-07-3-83, Work Project L-9809.

content than the  $P_1$  tips from which they had originally come. This indicates an auxin production by these roots.

The experiment was continued with another series of roots ( $P_2$ ) from which 10-mm. tips were taken. These tips contained on the average  $48.6 \times 10^{-6}$  gamma indoleacetic acid equivalents. The roots from these tips after one week contained on the average  $88 \times 10^{-6}$  gamma, thus showing an increased auxin content. The tips of this  $P_3$  series, containing  $50.1 \times 10^{-6}$  gamma of auxin per average root, developed little during subsequent subculturing and contained at the end of the week 36.5 gamma indoleacetic acid equivalents. The results of this experiment indicate, although not conclusively, the possibility of an auxin production in the isolated roots.

Experiments had to be carried out on a larger scale, using an improved nutrient. ADDICOTT and BONNER (1) improved the nutrient solution by adding both vitamin  $B_1$  and nicotinic acid. In this solution pea roots have been subcultured for 34 consecutive weeks. Only the bases were analyzed for auxin and the tips were subcultured. Even after 34 weeks of subculturing a large amount of auxin ( $1000 \times 10^{-6}$  gamma indoleacetic acid equivalents per average root base) was found to be present, and there is no reason to believe that later cultures would be devoid of it. In table 2 the results of an analysis covering 20 weeks are given. It should be noted that this table is composed of several experiments which together cover the entire range from 0 to 20 weeks of subculture. No data were available for the 15th, 16th, and 17th weeks; the auxin content at this stage was estimated to be equal to the average of the available data for  $P_1$  to  $P_{20}$ . A total amount of auxin  $2043 \times 10^{-6}$   $\gamma$  indoleacetic acid equivalents was extracted from the bases during the twenty successive weekly transfers (or  $1737 \times 10^{-6}$   $\gamma$  if the 15th, 16th, and 17th weeks are not counted). The average amount of auxin extracted from the initial root tip ( $P_0$ ) is  $232 \times 10^{-6}$  indoleacetic acid equivalents. This shows that an auxin amount nine times larger than that present in the initial tip can be extracted from roots grown from this tip. These results were obtained when the bases as well as the initial tips ( $P_0$ ) were extracted in the usual manner (8, 9, 11) by standing in ether for 1-3 days. In a special experiment in which initial tips were extracted in a Soxhlet extractor, an auxin amount as high as

$720 \times 10^{-6}$   $\gamma$  indoleacetic acid equivalents was once found for  $P_0$  (table 2). Thus even if initial tips were exhaustively extracted in a Soxhlet apparatus they still contained only about 30 per cent of the total amount of auxin extracted in the ordinary way from the bases of twenty consecutive subcultures. In view of the fact that  $P_{34}$  roots

TABLE 2

TOTAL AMOUNT OF AUXIN PER AVERAGE ROOT IN  $10^{-6}$  GAMMA INDOLEACETIC ACID EQUIVALENTS, DURING SUBCULTURING. EXCEPT FOR  $P_0$ , ONLY ROOTS WITHOUT THEIR TIP WERE ANALYZED. S INDICATES EXTRACTED IN A SOXHLET EXTRACTOR; E INDICATES ESTIMATED VALUE

CULTURE	DATE	CONTENT	AVERAGE	CULTURE	DATE	CONTENT	AVERAGE
$P_0$ (initial tip)	Aug. 2, 1938	230	232	$P_8$	Apr. 10, 1939	298	298
	May 9, 1939	201		$P_9$	Mar. 23, 1939	150	161
		265			Apr. 10, 1939	152	
	May 16, 1939	481 S 720 S			Apr. 17, 1939	180	
$P_1$	Aug. 9, 1938	39	39	$P_{10}$	Mar. 28, 1939	130 102	119
$P_2$	Aug. 16, 1939	54	53	$P_{11}$	Apr. 17, 1939	126	91
	Sept. 1, 1938	67			Apr. 10, 1939	85	
	Sept. 17, 1938	55			Apr. 17, 1939	85	
	Apr. 13, 1939	35		Apr. 28, 1939	102		
$P_3$	Sept. 30, 1938	38	93	$P_{12}$	Apr. 28, 1939	19	48
	Apr. 17, 1939	147		$P_{13}$	May 5, 1939	77	40
$P_4$	Apr. 28, 1939	27	40	$P_{14}$	May 5, 1939	40	
		47		$P_{15}$	Mar. 28, 1939	226	226
		52		$P_{16}$			102 E
$P_5$	May 5, 1939	35	40	$P_{17}$			102 E
		33		$P_{18}$	Apr. 17, 1939	115	115
		46		$P_{19}$	Apr. 28, 1939	78	78
$P_6$	Jan. 24, 1939	145	134	$P_{20}$	May 5, 1939	131	131
	Mar. 23, 1939	142					
$P_7$	Mar. 28, 1939	115 31	31				
Total for 20 bases. . . . 2043							

extracted in a Soxhlet apparatus yielded  $1000 \times 10^{-6}$   $\gamma$  (see following), it is likely that if the other bases also were extracted in the Soxhlet apparatus this percentage would be considerably smaller.

A small number of roots were left that had been subcultured for 34 weeks. The bases of fifteen such roots, each weighing on the average 18.9 mg., were extracted in the Soxhlet apparatus for 30 hours. The extract was taken up in 0.3 cc. of 1.5 per cent agar and gave a curvature of  $7.3^\circ$  in the *Avena* test. The blank control did not give

any auxin curvature ( $+0.8^\circ$ ) and a control having 55  $\gamma$  indoleacetic acid per liter curved  $7.1^\circ$ . From this the average auxin content of the average  $P_{34}$  base was calculated and found to be  $1000 \times 10^{-6}$   $\gamma$  indoleacetic acid equivalents, which is a surprisingly large amount of auxin for roots.

### Discussion

These results show that auxin is produced in roots growing in vitro. It seems probable by analogy with coleoptiles, stems, etc., that in these roots auxin also disappears, which may be due either to auxin utilization during growth or to destruction (12). Thus the auxin found by extraction may be the balance of auxin produced and auxin lost. It is generally assumed that auxin is produced

TABLE 3  
EFFECT OF DECAPITATION ON FORMATION OF SIDE ROOTS  
 $P_2$  ROOTS CONTINUED FOR ANOTHER WEEK

	DECAPITATED	INTACT
Average length of main root.....	55.9 mm.	78.5 mm.
Average number of side roots.....	3.8	1.3
Average total length of side roots.....	16.5 mm.	4.3 mm.
Number of roots.....	42	45

in the root tip because it has been observed that it is given off by the excised tip. In a paper dealing especially with this problem (10) it has been shown that the auxin given off by the tips of germinating pea roots is not produced, but is present in the tip when the latter is cut from the root. The fact, therefore, that auxin in relatively large amounts is given off by excised root tips of germinating peas cannot be construed to mean that auxin is produced by such tips. Another argument which has been used in favor of auxin production in the root tip is that a higher concentration is found in the tip than elsewhere in the root (4, 6, 11). This, however, may be due to an auxin accumulation in the tip (6). The following experiment tested this point.

Roots at the end of the second week of subculturing ( $P_2$ ) were decapitated and put back in fresh nutrient solution for a further week. As shown in table 3, decapitation greatly promotes the development of side roots (7). At the end of the week the roots were divided into two groups according to their having developed side

roots or their failure to do so. Table 4 shows that the original base weighed on the average 11.4 mg. At the end of the week the 192 roots which had developed side roots weighed on the average 23.2 mg., whereas the 198 roots which failed to develop side roots weighed 10.9 mg., which is approximately the same as the original base. The auxin content of the original base was  $66.6 \times 10^{-6} \gamma$  on the average. After one week the roots which had developed side roots contained  $27.8 \times 10^{-6} \gamma$  per root and those without side roots  $86.4 \times 10^{-6} \gamma$  indoleacetic acid equivalents (average 57.1). This was rather the reverse of what could have been expected on the basis of an increased auxin production due to an increased number of new root tips. It should be remembered, however, that the auxin extracted gives only the balance of auxin produced and lost and that the latter factor

TABLE 4  
AUXIN CONTENT IN  $10^{-6}$  GAMMA PER AVERAGE  
ROOT DURING DEVELOPMENT OF SIDE ROOTS

Decapitated P <sub>2</sub> roots (11.4 mg.)	66.6
One week later:	
192 with side roots (23.2 mg.)	27.8
198 that failed to develop side roots (10.9 mg.)	86.4

probably was larger in the case where side roots were formed on account of their faster growth. On the other hand, AMLONG (2) reported evidence indicating that if the root tip of germinating *Vicia* is removed the auxin content of the remaining root is decreased. NAGAO (5) recently reported that if excised root tips were placed on nutrient agar they began to grow and produce auxin. VAN OVERBEEK (10) showed that the auxin which diffuses out of the excised root tips into dextrose agar is present when the tip is cut off.

A factor of uncertainty in this type of experiment with roots is the question of the possible presence of an inactive auxin precursor. Since we cannot as yet test quantitatively for this factor, we do not know how much of it is present in the initial root tip. Neither do we know how easily this substance is converted into active auxin. One could imagine that in pea roots, even under the condition of ether extraction, an equilibrium exists between auxin precursor and active auxin. This could explain why Soxhlet extraction, where roots and extract are separated, yields more auxin than ordinary extraction.

From the experiments presented here it can be concluded that auxin is produced in isolated pea roots grown in vitro under sterile conditions. This may be due to either one or both of two things: An active synthesis of auxin from the nutrient medium (inorganic salts, sugar, thiamin, nicotinic acid) or the gradual activation of an auxin precursor present in the initial tip and "carried along" during sub-culturing.

### Summary

1. Evidence is presented which shows that auxin is produced in isolated pea roots cultivated in vitro under sterile conditions. The roots were cultured in such a way that at the end of each week the culture was continued with the 10-mm. tip only. The basal parts of the roots, that grew approximately 700 mm. per week, were discarded. The total amount of auxin extracted from these discarded bases during 20 weeks of culture was nine times as much as that extracted from the initial tip from which the cultures originated.

2. Even at the end of 34 weeks of culturing, roots were found to contain as much as  $1000 \times 10^{-6}$   $\gamma$  indoleacetic acid equivalents of auxin per root.

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## VERNALIZATION OF MARQUIS WHEAT AND OTHER SPRING CEREALS

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 507

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(WITH SIX FIGURES)

### Introduction

In 1933, WHYTE and HUDSON (22) presented a translation and compilation of the literature on vernalization of plants. A bulletin (9) appeared in 1935 which summarized the research on this subject in the Soviet Union and twenty-six other countries. Many aspects of vernalization, theoretical and practical, have recently been discussed by WHYTE (21).

There is great diversity of opinion with respect to the reaction of spring cereals to vernalization. Data collected by FOKEEV and VYROV (3, 4) from various places in the U.S.S.R., where extensive use of vernalization has been made, showed 2 to 4 days earlier emergence of vernalized seedlings from the soil. Vernalization stimulated root development, which in turn led to increased development of green parts. The thicker stand, which was maintained throughout the vegetative season, reduced weed growth. At harvest vernalized plants were on the average 9.5 cm. taller. Vernalized grain began earing 2.5 days earlier than controls. In dry districts there was a recorded increase in grain yield of 28.6 per cent as compared with 17-18 per cent in other districts. The quality of the wheat grain was also improved. ZENJUK (24) grew vernalized wheat at the Minsk Marshland Station to determine the acceleration of maturity. In later sowings (May 3) vernalization accelerated the onset of all developmental phases, whereas in early sowings the difference was evident only prior to ear emergence.

VAN HOEK (20), working in Holland, vernalized spring wheat for 7 days and for 16 days, at 3°-6° C. The 16-day treatment was the more successful, resulting in an acceleration of earing of 3 days and of maturity of 1-4 days. GMELIN (6), also working in Holland and

using the same variety of wheat as VAN HOEK, could obtain no acceleration of maturity. In Rumania, PIESCU (16) found that vernalized spring varieties headed at the same time as controls.

In the United States, MCKINNEY *et al.* (15) grew vernalized Marquis wheat at Langdon, North Dakota, 20 miles south of the Canadian boundary. No decreases in time to mature were obtained, but treatment for 10 days gave average increases in yield of 19.07 per cent over unchilled, ungerminated controls. TAYLOR and COFFMAN (19) vernalized Isogold, a variety of spring oats. Treatment retarded heading by 2 days and gave a decrease of 37 per cent in yield. This decrease is ascribed to less germination, since the stand in the vernalized plot for a period of 4 years averaged only 51 per cent of that in the untreated plots.

Referring to experimental data, KONSTANTINOV (10) asserts that although vernalization of cereals, maize, millet, sunflower, Sudan grass, and other crops is widely used in all parts of the Soviet Union, the efficacy of vernalization is closely connected with the region and after-sowing conditions. The cold spring of southeast Russia is rather unfavorable for vernalized sowings, or at least less favorable than the warm, moist, prolonged spring of certain other regions. The efficacy of vernalization varies also with the time of sowing, later sowings being more beneficial.

The chief edaphic difference between the early and late plantings, particularly at high latitudes, would be soil temperature, which would show an increase as the spring advanced. The preceding results therefore point to a possible correlation between soil temperature and efficacy of vernalization.

The phasic development theory assumes that plants can enter upon the next stages of development, given appropriate conditions, only when the previous stage has been completed. It is therefore of special importance to be able to determine whether a certain stage has been completed. Assuming that vernalization produces a change in the physico-chemical state of the protoplasm, BASSARSKAJA (1, 2) was able to detect the completion of the thermo-stage by the precipitation of Prussian blue within the tissues of the vernalized embryo upon addition of ferric chloride and potassium ferrocyanide. RICHTER (18) and GAVRILOVA (5) have used a staining technique

based upon the displacement of the iso-electric point of the tissues of the embryo to determine completion of the thermo-stage in cotton and wheat. A modification of the microchemical technique used by these investigators was employed in the diagnosis of vernalization of Marquis wheat.

In the following experiments, which were begun in 1934, the reaction of vernalized spring wheat, oats, barley, and rye with respect to vegetative period, yield, photoperiodic response, and growth at various soil temperatures was investigated. A preliminary attempt to use microchemical methods to diagnose the vernalized state is also described.

#### General method and terminology

Methods of vernalization are based primarily on the researches of LYSENKO (13). The general procedure for vernalization of all cereals consists in germinating the seeds for 24 hours at 15°–20° C. and then transferring the germinated grain to low temperatures for a period, allowing the young plant to continue development without increasing in size.

For convenience a formula is employed to express the combinations of factors employed in the vernalization treatment. The factors and their units are: (1) length of time of chilling in days; (2) temperature during chilling in degrees Centigrade; (3) amount of water added in percentage of original air-dry weight of the seed. For example, the formula 8:3:60 means that the seed was chilled for 8 days at 3° C. following germination, the water added being 60 per cent of the original air-dry weight of the seed.

The vernalization method used consists of four parts:

1. The amount of water was added to the seeds in petri dishes.
2. The imbibed seeds were allowed to germinate until radicles had pierced the seed coats of a few grains (usually within 24 hours at 15°–20° C.).
3. The germinated seeds were placed in the chilling chamber for the number of days indicated.
4. The covers of the petri dishes were removed and the seeds aerated for a few seconds each day.

Any departures from this method, necessary in the treatment of large quantities of seed, are noted in the text.

## Investigation

EFFECT OF VERNALIZATION ON LENGTH  
OF VEGETATIVE PERIOD AND YIELD

## A. FIELD EXPERIMENTS

Investigations covering the period 1934 to 1937 were conducted at Saskatoon, Saskatchewan ( $52^{\circ}$  N lat.,  $106^{\circ}$  W long.), while the work of 1939 was performed at Chicago, Illinois.

TABLE 1

TREATMENT, HARVEST DATA, ETC., FOR FIELD EXPERIMENTS, 1934-1939

YEAR	GRAIN	VERNALIZATION FORMULA	WEIGHT OF SEED TREATED	AREA SOWN	TREATMENT OF CONTROLS	ACCELERATION OF FLOWERING (DAYS)	ACCELERATION OF RIPENING (DAYS)	INCREASED YIELD DUE TO VERNALIZATION	DATE OF PLANTING
1934	Marquis wheat	5	30 gm.	Five rows	Unchilled, ungerminated	See table 2			May 5
	O.A.C. barley	7	"	4' long	"				"
	Hannchen barley	13	"	"	"				"
	Banner oats	16:3:33	"	"	"				"
	Victory oats	17	"	"	"				"
1935	Marquis wheat	18	"	"	"				"
1935	Marquis wheat	23	"	"	"				"
1935	Marquis wheat	12:3:33	300 lb.	5 acres	"	o	o	21.04%	May 6
1936	Marquis wheat	12:3:33	60 lb.	1 acre	"	o	o	14.04%	May 7
1937	Marquis wheat	12:3:33	60 lb.	1 acre	(a) Unchilled, ungerminated	.....	.....	.....	May 9
	Banner oats	12:3:33	20 lb.	1/4 acre	(b) Germinated	.....	.....	.....	May 9
					As for wheat, 1937	.....	.....	.....	May 9
1939	Marquis wheat	8:3:60	20 gm.	Seven rows 5'3" long	Germinated	2.3	.....	.....	July 3

Chilling: 1935, in room cooled by snow; others by mechanical refrigeration.

Planting: 1934, 1939, by hand; others by seed drill.

Location: 1939, in Chicago; others in Saskatoon, Sask.

The formula used during the years 1934 to 1937 was that of LYSSENKO, with respect to amount of water added and temperature during treatment. Various lengths of treatment (table 1) were used in 1934 in an attempt to determine the optimum combination of the several vernalization factors used. The formula used in 1939, 8:3:60, was the one developed in 1938 which gave the greatest acceleration of flowering of Marquis wheat grown in the greenhouse during that year.

A modification of the method just given was necessary to vernalize large quantities of seed. The water was added in three equal amounts, 4 hours apart, and the seeds were stirred periodically to insure uniform moistening.

In 1934 the chilling chamber was a basement room, snow serving effectively to maintain an average temperature of  $3^{\circ}$  C. During chilling, the grain, which had been spread in a layer 6 inches deep on the floor, was mixed thoroughly once a day to insure adequate aeration. The 60-pound and 20-pound lots treated in 1935, 1936, and 1937 were chilled in bags which allowed a relatively free access of air. In all cases the treated grain was sown, without being dried, on the day it was removed from the refrigeration room. When more than one formula was used all treatments were concluded at the same time.

When the seed drill was used, setting of the drill at 2 bushels per acre and 1 bushel per acre sowed equal dry weights of vernalized and ungerminated, unchilled control grain, respectively. The setting had to be increased slightly beyond 2 bushels in the case of the germinated control.

In the 1935 and 1936 experiments the plots were sampled by harvesting 50 areas of 1 square yard each, taken in regular order throughout the plots. The field plots of treated and control grain were one-quarter mile long and located side by side. The soil conditions in the plots were practically identical.

The experiments of 1934 to 1937, in which 33 per cent water was added to the original dry weight of the seed, gave little acceleration of maturity of Marquis wheat, Banner and Victory oats, and O.A.C. barley, regardless of duration of treatment. In the case of Hannchen barley, however, 5:3:33 and 13:3:33 both resulted in a 7-day advance in flowering (table 2). The formula 8:3:60, used in 1939, accelerated flowering of Marquis wheat in the field by an average of 2.3 days.

Increases in yield were general, amounting to 100 per cent over control in the case of Banner oats, vernalized according to the formula 23:3:33, in the small-scale trial of 1934. The large-scale sowings of 1935 and 1936 gave yield increases by vernalized Marquis wheat of 21.04 and 14.04 per cent, respectively, over the untreated controls. In 1935 the vernalized Marquis wheat showed an increase

in height of 7.2 per cent, an increase in number of fertile spikelets per head of 5.0 per cent, an increase in average kernels per head of 2.5 per cent, and an increased 1000-grain weight of 18.64 per cent.

Drought during the spring and summer of 1936 resulted in a smaller increase in yield due to vernalization than in 1935, when growing conditions were more favorable. Vernalized Marquis wheat did not appear to have any greater or less resistance to the extreme drought of 1937, which caused complete crop failure. In 1939 a week

TABLE 2

ACCELERATION IN DAYS TO FLOWER, TO RIPEN, AND INCREASED YIELD DUE TO VERNALIZATION. FIELD EXPERIMENT, 1934

DAYS VERNAL- IZED	ACCELERATION IN DAYS TO FLOWER OVER CONTROL					ACCELERATION IN DAYS TO RIPEN OVER CONTROL					PERCENTAGE INCREASED YIELD OVER CONTROL				
	MARQUIS WHEAT	O.A.C. BAR- LEY	HANNCHEN BARLEY	BANNER OATS	VICTORY OATS	MARQUIS WHEAT	O.A.C. BAR- LEY	HANNCHEN BARLEY	BANNER OATS	VICTORY OATS	MARQUIS WHEAT	O.A.C. BAR- LEY	HANNCHEN BARLEY	BANNER OATS	VICTORY OATS
5.....											20.8	64.3	74.3	70.4	50.0
7.....	-1.3	-1.1	7	-1.4	1.0	1.0	-1.1	5	-1.2	-1.1	14.6	52.3	37.3	50.0	59.3
13.....	-1.0*	1.1	7	1.1	1.0	1.0	1.1	5	1.0	1.1	5.2	54.0	40.1	85.0	37.3
16.....	-1.3	1.1	7	1.1	1.1	1.1	1.1	4	1.0	1.1	3.1	3.3	20.4	70.3	23.1
17.....	-1.3	1.1	5	1.0	1.1	1.1	1.1	2	1.1	1.0	7.3	31.0	2.3	48.1	18.8
18.....	3	1.0	6	1.1	1.1	1.1	1.1	2	1.0	1.1	0.4	51.0	20.6	62.0	25.0
23.....	2	0	3	1.1	0	0	1.1	2	1.0	1.1	27.1	74.1	37.3	100.0	28.1

\* Minus sign indicates retardation in flowering or ripening.

of hot dry weather immediately following planting reduced germination of vernalized seeds of Marquis wheat 28.5 per cent more than the reduction in germination suffered by the control grain. At the end of this week the plots were watered and watering was continued periodically throughout the experiment. In spite of the greater reduction in germination, the vernalized plants responded to irrigation by producing 47.5 per cent more heads than controls in 87 days, the period of the experiment.

#### B. GREENHOUSE EXPERIMENTS

*Experiment 1.*—The results of the experiments concerning the photoperiodic response of various vernalized cereals, described in a later section, indicated the necessity for developing a formula for treatment, specific for each cereal. An attempt was made at Chicago

during the summer of 1938 to determine the vernalization formula for Marquis wheat which would give the shortest vegetative period. The experiment was begun June 21 and terminated August 26.

The possible combinations, 125 in all, of the following vernalization factors were employed:

1. Length of vernalization period: 5, 8, 11, 14, 17 days.
2. Temperature during vernalization: 3°, 5°, 7°, 10°, 13° C.
3. Water added: 20, 30, 40, 50, 60 per cent.

Two complete series of these various combinations were run as indicated in the following paragraphs. Forty-gm. lots of Marquis wheat were treated, using the procedure outlined at the beginning of this report and the 125 combinations of factors just given.

Series A consisted of lots of seed started at the same time and removed from the mechanical refrigerators at the end of 5, 8, 11, and 14 days. The removals and plantings were on June 27, June 30, July 3, July 6, and July 9. Series B consisted of lots of seed placed in the refrigeration chambers at such times that all the periods of treatment, including 17 days, were completed on July 9. Thus only series B included seed vernalized for 17 days.

Three plants were grown in 6-inch pots filled with loam soil. The control was grain to which water equal to 40 per cent of the dry weight had been added and allowed to germinate 24 hours at 20° C. No control over the temperature of the greenhouse was attempted. This rose nearly every day to 100° F. and fell at night to 70° F.

1. Effect of drying seed subsequent to treatment.—The percentage germination of the grain prior to vernalization was 94.75 and immediately after treatment 95.0. After being air-dried for 3 days following vernalization the germination was 86.0 per cent. Drying the vernalized grain decreased germination by approximately 9.5 per cent.

2. Length of vegetative period.—In table 3 only those combinations of length of vernalization, temperature, and percentage water added which resulted in a decrease in length of the vegetative period, compared with controls, in both series A and B are given. No results complying with this condition were obtained with 14-day treatment although decreases in time to flower were obtained in several cases in series A.

The combination of vernalizing factors which gave the earliest flowering of Marquis wheat under greenhouse conditions was 8:3:60. This formula gave an acceleration of 24 days in series A and 18 days in series B, or an average of 21 days in comparison with controls. In both cases the grain was ripe (the head showing no green color) or would have ripened in 50 days after planting. The formulas 8:10:50 and 8:13:20 produced an acceleration in flowering of 14.5 and 13

TABLE 3

COMBINATIONS OF VERNALIZING FACTORS GIVING DECREASES IN VEGETATIVE PERIODS IN SERIES A AND B, 1938, GROWN IN GREENHOUSE

VERNALIZATION FORMULA	SERIES A						SERIES B					
	DAYS FOR TREATED PLANTS TO			CONTROL*		ACCELERATION IN DAYS TO FLOWER DUE TO VERNALIZATION	DAYS FOR TREATED PLANTS TO			CONTROL*		ACCELERATION IN DAYS TO FLOWER DUE TO VERNALIZATION
	HEAD	FLOWER	RIPE	HEAD	FLOWER		HEAD	FLOWER	RIPE	HEAD	FLOWER	
5:3:50....	39	42	60	47	47	5	41	41	.....	48	49	8
5:5:50....	43	40	.....	47	47	1	40	40	.....	48	49	9
5:13:60....	43	45	60	47	47	2	30	32	.....	48	49	17
8:3:50....	43	44	.....	55	50	12	40	41	.....	48	49	8
8:3:60....	31	32	50	55	50	24	30	31	†	48	49	18
8:10:40....	47	48	.....	55	50	8	33	33	.....	48	49	16
8:10:50....	44	44	.....	55	50	12	30	32	.....	48	49	17
8:13:20....	45	40	.....	55	50	10	30	33	.....	48	49	16
8:13:60....	48	48	.....	55	50	8	41	41	.....	48	49	8
11:3:30....	48	48	.....	52	53	5	33	34	.....	48	49	15

\* No controls ripe August 26, when experiment was terminated.

† Nearly ripe in 48 days.

days, respectively; 17:5:60 and 17:7:40 gave an acceleration of 17 days in flowering compared with their controls, in the one attempt using 17-day vernalization. A decrease in temperature during treatment apparently can be counteracted by an increase in moisture content of the seed, within limits.

*Experiment 2.*—Marquis wheat was vernalized using the formula 8:3:60, developed in the preceding experiment, and grown in 8-inch pots in the laboratory at Saskatoon. The natural daylight was supplemented by electric lights of 200 watts, suspended 12 inches above the plants, to give a total of 15 hours of light per day. Soil temperature readings were taken twice a day, at 9 A.M. and 4 P.M. The average morning reading was 15° C. and the average afternoon soil

temperature was 20° C. The date of sowing was October 23. The number of vernalized plants was twenty-four and controls twelve.

The vernalized plants (a) emerged 2 days earlier; (b) formed each of the seven leaves at least 2 days earlier; (c) headed an average of 5.5 days earlier; (d) flowered an average of 7 days earlier; (e) ripened 4 days earlier; and (f) had a 1000-grain weight 10.37 per cent greater than the controls.

*Experiment 3.*—The formula 8:3:60 was used to vernalize Marquis wheat at Chicago during the summer of 1939. Three vernalized plants were grown in each of eighteen 6-inch pots and three control plants in each of twenty-one pots.

The wheat, the previous history of which was unknown, did not respond to treatment as did that of the previous year. In the term of the experiment, July 3 to September 3, 46.4 per cent of the treated plants had flowered in an average of 53 days. Of the controls 44.4 per cent flowered in an average of 53.5 days. Treatment increased the number of flowerings but accelerated maturity by only 0.5 day.

In a repetition of the experiment begun August 3, the vernalized grain flowered in an average of 49.7 days and the control in 52.3 days. The acceleration of flowering resulting from vernalization was 2.6 days. In both the 1938 and 1939 experiments the flowering time was shorter and the effects of vernalization more pronounced in later plantings.

The acceleration of flowering caused by vernalization of from 8 to 18 days in 1938 and but 0.5 to 2.6 days in 1939 when two different samples of vernalized Marquis wheat were grown in the greenhouse under similar conditions is perhaps due to differences in the histories of the wheats prior to treatment. The wheat used in 1939 may have been naturally vernalized to some extent during ripening and thus did not respond to the same degree to further vernalization. The result of the 1939 experiment suggested the problem of vernalization of spring wheat prior to reproductive maturity, which is being investigated at the present time.

#### PHOTOPERIODIC RESPONSE OF VERNALIZED SPRING CEREALS

Many workers in photoperiodism have determined that some cereals respond to increased photoperiods by an acceleration in

flowering. An experiment was conducted in 1934 to test the comparative response of vernalized and unvernallized spring cereals to continuous illumination.

Marquis wheat, Banner oats, O.A.C. barley, and Prolific rye were vernalized for periods of 5, 7, 13, 16, 17, 18, and 23 days using a temperature of 3° C. and 33 per cent added moisture. Five seeds of each set of cereals were planted in each of two 10-inch pots, filled with loam soil. The plants were grown in a greenhouse the temperature of which was uncontrolled. One complete set (two pots of each

TABLE 4  
RESPONSE OF VERNALIZED AND UNVERNALLIZED SPRING CEREALS  
TO INCREASED PHOTOPERIOD

GRAIN	VER- NALI- ZATION FORMU- LA	DAYS TO FLOWER						ACCELERATION DUE TO VERNALIZATION			DECREASE IN DAYS TO FLOWER DUE TO CONTINU- OUS ILLU- MINATION	
		GREENHOUSE				FIELD		GREENHOUSE		FIELD	VER- NAL- IZED	CON- TROL
		VER- NAL- IZED 24 HOURS LIGHT	CON- TROL 24 HOURS LIGHT	VER- NAL- IZED NOR- MAL LIGHT	CON- TROL NOR- MAL LIGHT	VER- NAL- IZED	CON- TROL	CON- TINU- OUS LIGHT	NOR- MAL LIGHT			
Marquis wheat.	23:3:33	36	37	46	50	59	61	1	4	2	10	13
Banner oats....	13:3:33	35	36	46	49	67	68	1	3	1	11	13
O.A.C. barley..	13:3:33	35	35	54	58	65	66	0	4	1	19	23
Prolific rye.....	13:3:33	29	29	36	40	.....	.....	0	4	.....	7	11

cereal for each vernalizing formula) was exposed to normal illumination and another complete set to continuous illumination. Daylight was supplemented by electric lights of 500 watts suspended 4 feet above the tops of the pots.

The plantings, seven in all, were completed on May 5, 1934. Two controls were used, one of seeds germinated for 24 hours at 15° C. and the other of ungerminated, unchilled seeds. The experiment was conducted at Saskatoon.

In all cases the germinated and ungerminated controls flowered simultaneously. TAYLOR and COFFMAN (19) report a similar result in their experiments with spring oats.

The treatments which gave the greatest acceleration in time to flower (extrusion of anthers), compared with controls grown in the same photoperiod, are given—together with flowering data—in

table 4. Data from the field experiment of 1934 are included for comparison. The 13:3:33 treatment resulted in the shortest vegetative period for Banner oats, O.A.C. barley, and Prolific rye. Marquis wheat, however, flowered in the shortest time when vernalized according to 23:3:33.



FIG. 1.—A: O.A.C. barley grown in normal illumination. Left to right: control 18 days; vernalized 18 days; control 7 days; vernalized 7 days. B: Banner oats grown in normal illumination. Left, control; right, vernalized 13 days.

In each case the acceleration of flowering caused by vernalization, compared with controls grown under the same light conditions, was less in continuous light than in normal illumination. Untreated plants reacted more than treated plants to increased illumination. The cereal whose flowering was accelerated most (19 days when vernalized, 23 days when untreated), due to a photoperiod of 24 hours, was O.A.C. barley; the least responsive was Prolific rye. Other responses are given in table 4.

Grains were produced very sparingly, in some cases having as few as six kernels a head. An estimation of possible yield, however, could be obtained from the size and number of heads. Vernalization resulted in greater height, greater stem diameter, larger average num-

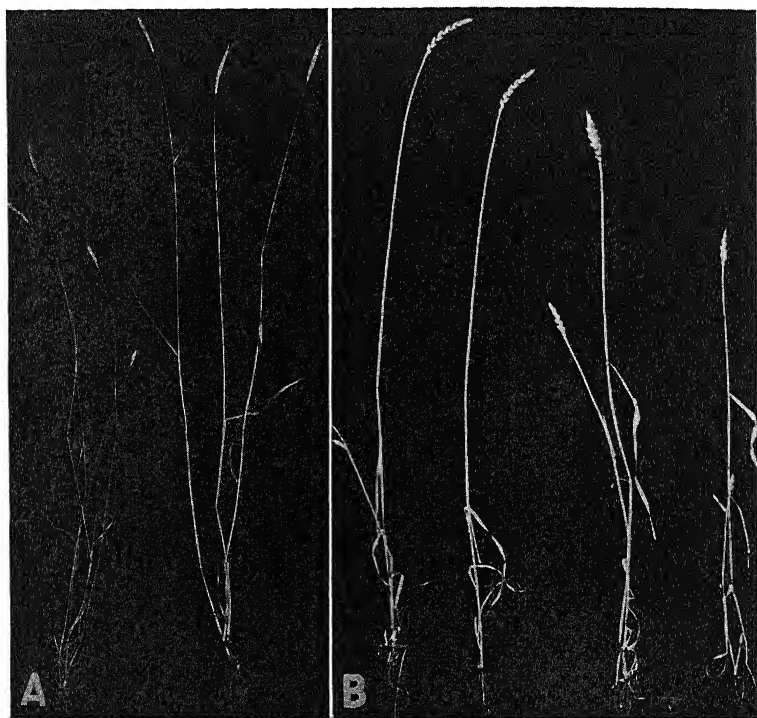


FIG. 2.—A: prolific rye grown in normal illumination. Left, control; right, vernalized 16 days. B: Marquis wheat. Left to right: vernalized 18 days, grown in continuous illumination; control 18 days, grown in continuous illumination; vernalized 18 days, grown in normal illumination; control 18 days, grown in normal illumination.

ber of tillers, and larger heads than controls when grown under both normal and continuous illumination (figs. 1, 2). Plants receiving 24 hours of light were taller than those grown under normal illumination, the increased height being due to lengthened internodes.

A comparison of time for flowering in the field and in the greenhouse under the same photoperiod indicates, for all cereals grown, that vernalization accelerated maturity more when the plants were

grown at the higher temperature of soil and air in the greenhouse. This possible correlation between response to vernalization and soil temperature was investigated in the experiments described in the next section.

#### SOIL TEMPERATURE IN RELATION TO VERNALIZATION

To determine whether a correlation existed between soil temperature and vernalization response, vernalized Marquis wheat was grown at Chicago at various soil temperatures during 1937, 1938, and 1939. The method for each year is given in table 5.

TABLE 5  
METHODS FOR GROWTH OF VERNALIZED WHEAT AT  
VARIOUS SOIL TEMPERATURES

YEAR	VERNALIZATION FORMULA	PLANT- ED	HAR- VESTED	INITIAL GROWTH	SOIL TEM- PERATURE (°C.)	REPLI- CATE POTS, EACH TEM- PERA- TURE	TOTAL PLANTS, EACH TEM- PERA- TURE	DURA- TION OF EXPERI- MENT (DAYS)
1937.....	11:3:40	July 1	Aug. 27	Five days at 26° C., then at tempera- tures given	22°, 33°, 44°	3	15	57
1938.....	8:5:40	July 9	Aug. 26	Given temperatures from the start	22°-44° in 2° intervals	3	15	48
1939.....	8:3:60	July 3	Sept. 11	As 1937	22°, 26°, 30°, 34°, 38°, 42°	5	15	70

The vernalized and control wheats were planted in loam soil in 2-gallon porcelain containers. Five plants were allowed to grow in each pot during the 1937 and 1938 experiments and but three in 1939. The containers were immersed to within 2 inches of their tops in water. The temperature of the water was thermostatically controlled with the exception of the tank at 22° C. The lowest temperature that could be obtained by using continuously running water from the water mains was 22° C., and the temperature of the water (and hence of the soil in the containers) rose at times to 24° and fell to 21° C. In the other soil temperature tanks the fluctuations were  $\pm 0.5^{\circ}$  C.

The temperature of the air in the greenhouse was uncontrolled and rose nearly every day to at least 100° F., falling at night to about

70° F. The plants were given sufficient water once a day to keep the soil in all pots moistened to approximately the same degree. More water had thus to be added to the pots at higher temperatures than at lower temperatures. Drains from the containers provided for removal of excess water.

Results of the three experiments were very similar, and only data for 1939 are tabulated in table 6. Significant differences are mentioned in the text.

TABLE 6  
HARVEST DATA, SOIL TEMPERATURE EXPERIMENT, 1939

SOIL TEMPERATURE °C.	VERNALIZED OR CONTROL	NO. PLANTS HARVESTED	AVERAGE DRY WEIGHT (GM.)			TOP/ROOT RATIO	GREATEST AVERAGE HEIGHT (IN.)	GREATEST AVERAGE ROOT LENGTH (IN.)	AVERAGE NO. OF TILLERS/PLANT	TOTAL HEADS IN 70 DAYS	AVERAGE HEADS PER PLANT	TIME FOR FIRST FLOWERING IN DAYS	AVERAGE TIME FOR FLOWERING IN DAYS	ACCELERATION OF FLOWERING DUE TO VERNALIZATION IN DAYS
			TOPS	ROOTS	TOTAL									
22...	V C	15	1.45	0.50	1.95	2.9	25.3	17.3	4.7	7	0.47	65	67.6	-1.3
		15	1.61	0.40	2.01	4.0	25.0	12.2	4.3	6	0.40	63	66.3	
26...	V C	15	1.10	0.21	1.31	5.5	22.7	12.1	3.7	12	0.80	48	59.8	4.3
		13	0.96	0.23	1.19	4.2	22.5	12.1	3.5	10	0.77	60	64.1	
30...	V C	12	0.38	0.07	0.45	5.4	19.6	10.1	2.5	3	0.25	52	58.3	2.3
		10	0.62	0.11	0.73	5.6	20.1	9.9	2.3	11	1.10	54	60.6	
34...	V C	14	0.29	0.04	0.33	7.2	16.3	5.4	1.9	6	0.43	48	53.0	2.0
		14	0.21	0.03	0.24	7.0	16.4	4.4	1.6	4	0.30	51	55.0	
38...	V C	15	0.15	0.03	0.18	5.0	12.4	3.3	1.4	4	0.27	52	55.5	6.5
		14	0.14	0.03	0.17	4.7	11.5	3.3	1.3	3	0.21	62	62.0	
42...	V C	8	0.10	0.028	0.128	3.6	10.9	2.9	1.3	.....	.....	.....	.....	.....
		8	0.06	0.013	0.073	4.6	7.2	2.3	1.4	.....	.....	.....	.....	

As a rule the average height of the vernalized grain, as measured from the soil level to the tip of the longest leaf of the leading tiller, was slightly greater than controls at all soil temperatures throughout the growing period. Figure 3 expresses graphically the average growth of treated and untreated wheat from the age of 5 days to 35 days at the various soil temperatures. The vernalized plants were 3.7 inches taller than the controls at 42° C. and showed a greater dry weight. Less dying of leaves occurred in the treated plants. In the 1937 experiment all control plants grown at 44° C. died within 35 days, but four vernalized plants survived the 57 days, and two headed

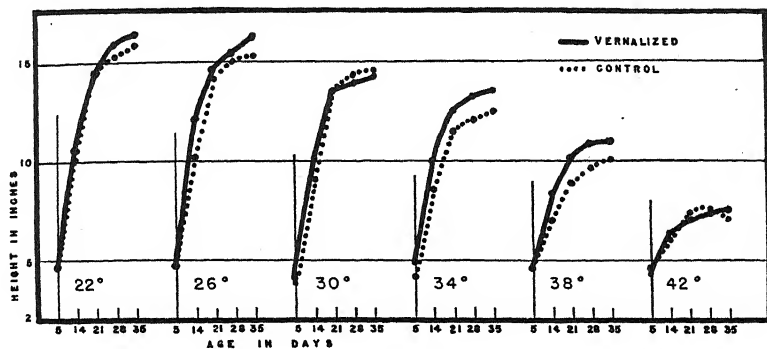


FIG. 3.—Average growth of vernalized and control Marquis wheat at soil temperatures of 22° to 42° C., from age of 5 to 35 days.

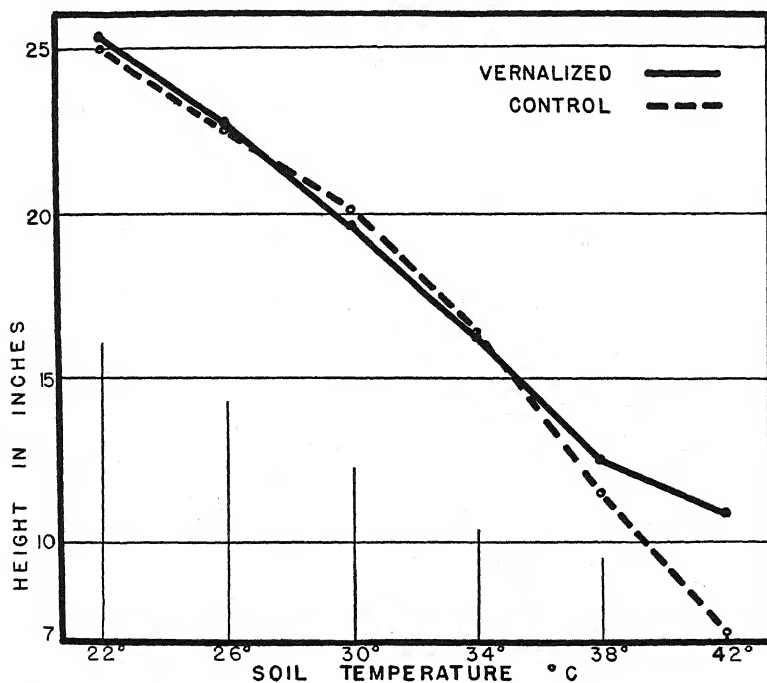


FIG. 4.—Heights of vernalized and control Marquis wheat grown at soil temperatures of 22° to 42° C., at age of 70 days.

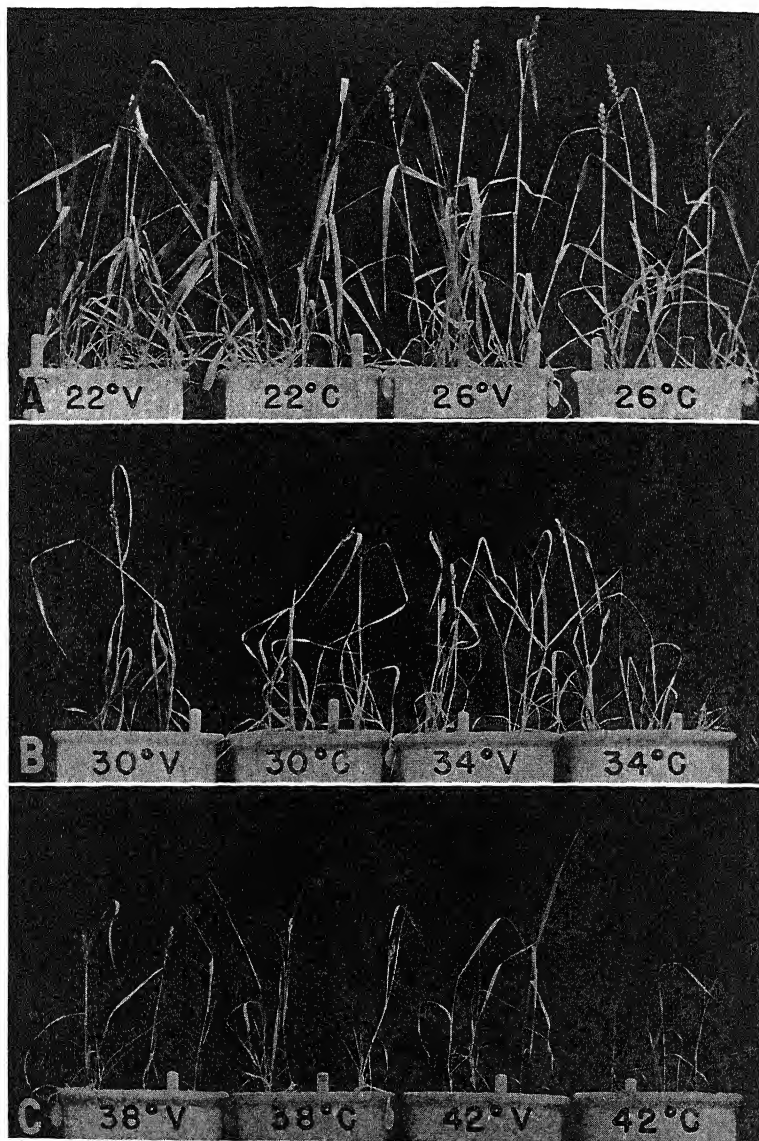


FIG. 5.—Representative plants of vernalized and control Marquis wheat grown at soil temperatures of 22° to 42° C., at age of 70 days. V, vernalized; C, control.

and produced grain. The height of the plants at 70 days, when they were harvested, is shown in figure 4, while figure 5 shows representative plants from each of the six soil temperatures.

The average greatest root length of treated plants was also greater than that of controls at all temperatures, although the comparative extent of the root systems varied somewhat. Roots decreased in length and extent with increasing soil temperatures.

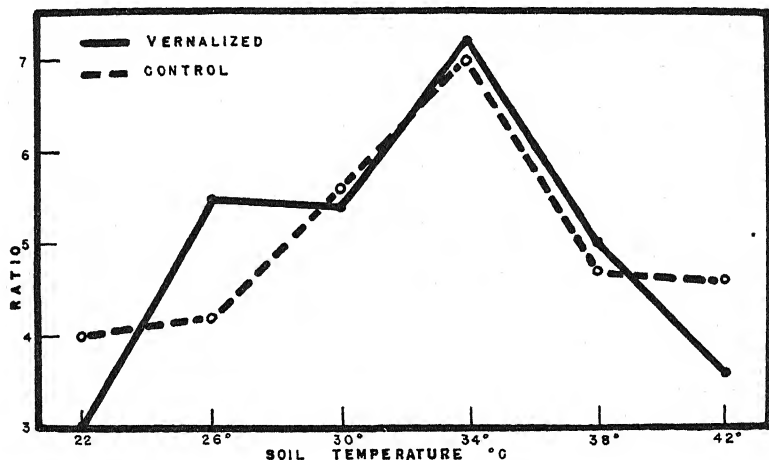


FIG. 6.—Top/root ratios of vernalized and control Marquis wheat grown at soil temperatures of 22° to 42° C., based upon dry weights at age of 70 days.

The number of tillers was greater on vernalized than on untreated plants, the average increase in the three experiments being 5 to 18 per cent.

No definite statement can be made from the results obtained as to the comparative dry weight behavior of the treated and untreated plants. Dry weights of both were less as temperatures increased. In both vernalized and unvernallized plants there was a rise in the top/root ratio as the temperature increased from 22° to 34° C., followed by a fall at higher temperatures (fig. 6). The high ratio indicates that the tops suffered comparatively less than the roots as the temperature rose to 34° C., but above this temperature the reverse is true.

The flowering behavior in 1937 and 1939 was similar. In 1937 the

control and vernalized plants flowered simultaneously in an average of 57 days when grown at a soil temperature of 22° C. At 33° C. the vernalized plants flowered in 48 days, the controls in 51 days. Two of the vernalized plants that survived at 44° C. flowered in 39 days. All control plants had died within 35 days. In 1939 both control and treated plants flowered in a shorter time as the soil temperature rose from 22° to 34° C. (table 6). Above 34° C. the flowering time was increased or earing prevented. The greatest acceleration of flowering resulting from treatment (6.5 days) occurred in plants grown at a soil temperature of 38° C.

In 1938 the vernalized plants flowered simultaneously at all soil temperatures from 22° to 40° C. The average time was 46 days. The controls had not headed within 48 days, and judging from appearance, would not have done so until at least 55 days. Vernalization accelerated maturity at all temperatures by approximately 10 days. A greater number of flowerings occurred, in the duration of the experiment, among vernalized plants grown at soil temperatures between 34° and 40° than at temperatures of 22° to 32° C.

The flowering behavior thus showed earlier flowering of both vernalized and control plants as the soil temperatures increased from 22° to 34° C.; and greater acceleration of flowering of vernalized plants over controls, or a greater number of flowerings in a given period, at higher soil temperatures.

The survival and flowering of some of the vernalized plants at a soil temperature of 44° C., their greater dry weight and smaller amount of dying of leaves at high temperatures than controls, indicate that—given adequate moisture—vernalized plants withstand high soil temperatures to a greater degree than untreated plants.

#### DIAGNOSIS OF THE VERNALIZED STATE

As advance in development of a plant is not always evidenced by morphological changes, it would be of benefit to be able to detect the qualitative changes which mark the completion of a phase by some microchemical method. A preliminary attempt, using modifications of methods employed by BASSARSKAJA (1, 2) and RICHTER (18), was made to detect completion of the thermo-stage (vernalization) in Marquis wheat seeds.

Marquis wheat was vernalized using 8:3:60 and the seeds were tested, employing the following techniques, after being chilled 4 days and again at completion of vernalization. The control was seed to which 60 per cent water had been added and allowed to germinate for 24 hours at 20° C.

a) Ferric chloride-potassium ferrocyanide method:

1. Sections of embryo cut at a thickness of 75  $\mu$ , using a freezing microtome.
2. Sections mounted using a gelatin fixative.
3. Slides treated with 5% ferric chloride for 3 minutes, rinsed thoroughly several times with distilled water, then treated for 3 minutes with 5% potassium ferrocyanide.

Treated in this way the vernalized and unvernallized seeds showed differences in reaction. As the seeds passed toward completion of the thermo-stage (vernalization), the color of the growing point changed from yellow, through green with areas of yellow, to a final dark blue. Tissues other than the growing point stained blue from the start.

b) Change of the iso-electric point:

1. Embryos sectioned and mounted as previously (crushing the embryo tissue on a slide smeared with fixative also proved faster and satisfactory).
2. Slides treated for 15 minutes with McIlwain's buffer solutions of pH 3.6 to 7.0 with intervals of 0.2.
3. Sections stained for 5 minutes in an aqueous solution formed by mixing equal volumes of a 1% solution of eosin and a 1% solution of methylene blue at the moment of staining.
4. After staining, slides rinsed in distilled water and returned to buffer solutions for 6 hours.
5. At conclusion of the 6 hours the pH of the buffers was determined, using a Coleman glass electrode apparatus.

At pH 5.32 sections from unvernallized seeds stained blue. At the end of 4 days of chilling the blue color was obtained at pH 5.13. When vernalization had been completed the embryos stained blue at pH 4.88. Thus, interpreting the results as does GAVRILOVA (5), the iso-electric point changed from pH 5.32 to pH 4.88 during vernalization.

### Discussion

The development of a plant (advance to reproductive maturity) consists of a continuous series of phases occurring in strict sequence, according to the theory of vernalization or phasic development. The conditions required for the phases differ and the progress of each phase depends on an entire complex of habitat factors. At least four such phases have been reported (9, 21). In cereals the first or thermo-phase requires a relatively low temperature for its completion. Light is unimportant during this phase. The second or photo-phase requires a high temperature and a long day.

The aim of vernalization treatment is to induce the young embryo, which has just started growth, to complete the first phase of development during that period when its nutrition is least dependent upon external factors. The progress of the thermo-phase depends on a complex of seed moisture, temperature, and aeration. The experiments with Marquis wheat indicate that within certain limits a higher temperature may compensate for a moisture content below the optimum.

Species and even varieties are specific in their requirements of vernalizing factors (21). Hannchen barley responded to 12:3:33 whereas the other cereals did not, the moisture content apparently being too low. Of 125 different treatments Marquis wheat responded most markedly to 8:3:60. Decreases in time to flower of Hannchen barley and of Marquis wheat agree essentially with the results of a number of other workers (3, 4, 20). The failure of some investigators (6, 15, 16, 19) to obtain acceleration of maturity when cereals were vernalized may possibly be attributed to an ineffective combination of vernalizing factors during treatment.

The majority of investigators obtained increases in yield when spring cereals were vernalized. Reported increases of between 15 and 20 per cent (3, 4, 15) were substantiated by my field trials which gave an increase of 21.04 per cent on 5-acre plots.

The findings by FOKEEV and VYROV (3, 4) that vernalization resulted in greater increases in yield compared with control grain in areas of drought are not in accord with the results of these experiments.

The results obtained in the experiments involving photoperiodic

responses of Marquis wheat agree with those of McKINNEY and SANDO (14), who found that Marquis wheat completes its life cycle quickly with a long photoperiod and a temperature of 70° F. or more throughout its life cycle. These workers also found that Marquis wheat formed seed very sparingly when grown in the greenhouse.

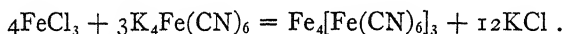
KOSTJUCENKO (11) reports that vernalized and unvernallized plants of spring wheat headed 5-9 days earlier in Hibiny (24-hour day) than in Pushkin (18-hour day). Continuous illumination in the experiment on photoperiod response resulted in earlier heading of vernalized plants by 7-19 days and of unvernallized plants by 11-23 days. The acceleration of flowering caused by vernalization was less in continuous light than in normal day length.

The response to vernalization is undoubtedly connected with the type of thermo-phase and the degree to which this phase has been completed prior to seed maturity (7, 8, 11, 12, 23), yet the response to treatment is definitely correlated with the after-sowing conditions and place of test. Thus the response to vernalization (shortening of the vegetative period) and length of the phase are not identical, for the after-sowing behavior of plants is governed by the environment, which has a different effect on the development of vernalized and unvernallized plants sown simultaneously. The Marquis wheat grown at various soil temperatures and the vernalized cereals grown simultaneously in the field and greenhouse in 1934 illustrate this point.

While the response obtained was not so simple as that reported by RAZUMOV and SMIRNOVA (17) for Bokhara barley grown in the continuous natural sunlight of the Arctic Circle in the greenhouse and in the field, the explanation is probably similar—the effect of temperature on the thermo- and photo-phase of the cereals. The thermo-phase of the unvernallized cereals was retarded at the higher temperatures, while the photo-phase proceeded more slowly at the lower temperatures. The photo-phase of the vernalized plants likewise proceeded more slowly at the lower temperatures than at the high; but because the thermo-phase had been completed during the vernalization treatment the higher temperatures resulted in a rapid completion of the photo-phase, giving an acceleration of 6.5 days in flowering compared with controls at a soil temperature of 38° C.

Not all developmental phases are perceptible morphologically, and not all morphological changes are a direct result of the transition of one phase to another. The morphological manifestations of those qualitative changes which constitute a development phase are subsequent in time to these changes, and the manifestations depend entirely upon the environmental conditions. From this standpoint the significance of the so-called phenological phases in recording the progress of development is practically annulled (21). The detection of biochemical changes within the plant, however, may serve to indicate the completion of a particular phase.

The formation of Prussian blue in the tissues of the embryo when treated with ferric chloride and potassium ferrocyanide is due to the reaction



The absence of Prussian blue in the cells of the growing point of the unvernallized seed may be due to two things: the ferric chloride does not penetrate into the cells or is reduced to ferrous chloride in the tissues (1, 2). The latter suggestion is confirmed by the formation of Prussian blue on the addition of potassium ferricyanide to the slides originally staining green. The conclusion is that vernalization produces a change in the oxidation-reduction potential of the cells. The shifting of the iso-electric point upon completion of vernalization indicates that the albumino-lipoids are likewise involved.

All that can be said with any degree of certainty is that some internal readjustments preceding morphological differentiation in the promeristem constitute the essentials of the thermo-phase (21). Although supplying little information regarding the exact nature of these changes, the diagnostic experiments suggest it is the physical and chemical properties of the protoplasm which are concerned, and as a matter of course, form the basis for diagnosis.

### Summary

1. The reaction of Marquis spring wheat to vernalization was studied through a period of 6 years. Other spring cereals investigated included O.A.C. and Hannchen barley, Banner and Victory

oats, and Prolific rye. Treatment was successful when snow or mechanical refrigeration was used to maintain chilling temperatures. Acceleration of flowering due to vernalization did not exceed 2.3 days when the cereals were grown in the field, with the exception of Hannchen barley which flowered 7 days and ripened 5 days in advance of controls. For plants grown in the greenhouse, the vernalization formula 13:3:33 (13 days chilling at 3° C., the water added being 33 per cent of the original air-dry weight of the seeds) resulted in an acceleration of flowering of 4 days in the case of O.A.C. barley and Prolific rye and of 3 days in the case of Banner oats. Of 125 different combinations of vernalization factors tested, 8:3:60 gave the greatest acceleration of flowering of Marquis wheat when grown in the greenhouse, the greatest average acceleration being 21 days. Increases in yield due to treatment amounted to 21.04 per cent on 5-acre plots. Increase in percentage yield due to treatment was less in time of drought than when growing conditions were favorable. Air drying vernalized seed reduced the percentage germination by 9.5.

2. Acceleration of flowering of Marquis wheat, O.A.C. barley, Banner oats, and Prolific rye due to vernalization was less when grown under continuous illumination than in normal day length, compared with controls receiving the same photoperiod. Increasing the photoperiod from normal day length to 24 hours shortened the vegetative period of untreated plants by 11-23 days and that of vernalized plants by 7-19 days.

3. Marquis wheat was grown at soil temperatures ranging from 22° to 44° C. Behavior showed earlier flowering of both vernalized and control plants as the soil temperature rose from 22° to 34° C., and a greater acceleration of flowering or a larger number of flowerings, due to vernalization, in plants grown at higher soil temperatures. While the dry weights of control and vernalized plants decreased as the soil temperature rose from 22° to 44° C., the top/root ratio rose to a maximum at 34°, falling to approximately the original value as the soil temperatures rose above 34° C. The growth of vernalized Marquis wheat in the greenhouse, with its higher temperatures, likewise resulted in a greater acceleration of flowering than when grown in the field.

4. Two microchemical methods were used to diagnose the vernalized state of Marquis wheat. Treatment of embryos with ferric chloride and potassium ferrocyanide showed a change in the ability of the growing point to reduce ferric chloride as vernalization progressed. A shifting of the iso-electric point of the embryo tissues from pH 5.32 to pH 4.88 upon completion of vernalization was detected by the use of McIlwain's buffers and staining with eosin and methylene blue.

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## CHROMOSOME NUMBER IN SOME TULIP HYBRIDS<sup>1</sup>

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### Introduction

During some studies on interspecific and intervarietal hybridization in the genus *Tulipa*, it developed that certain triploid varieties and species as well as a pentaploid species could be used as parents in the formation of hybrids. These hybrids are interesting in that the majority of them are aneuploids. HALL (7) has recently reported similar results with the same types of crosses, although he does not list the parents involved. He also points out that such aneuploid plants have not been previously reported either in species or in garden varieties, although *T. galatica* ( $2n=32$ ) might be placed in such a category (13, 23). However, HALL (7), reporting on some unpublished work of DARLINGTON and UPCOTT, considers the extra chromosomes in this form as supernumerary, and adds that the chromosome number is variable.

The successful hybrids involving either triploid or pentaploid parents are as follows:

*T. lanata* Regel. ( $2n=36$ )  $\times$  *T. tubergeniana* Hoog. ( $2n=24$ )

Inglescombe Yellow ( $2n=36$ )  $\times$  John Ruskin ( $2n=24$ )

John Ruskin ( $2n=24$ )  $\times$  Inglescombe Yellow ( $2n=36$ )

Clara Butt ( $2n=24$ )  $\times$  Inglescombe Yellow ( $2n=36$ )

*T. clusiana* D.C. ( $2n=60$ )  $\times$  *T. chrysantha* Boiss. ( $2n=48$ )

*T. chrysantha* ( $2n=48$ )  $\times$  *T. clusiana* ( $2n=60$ )

*T. clusiana* ( $2n=60$ )  $\times$  *T. stellata* Hook. ( $2n=24$ )

*T. clusiana* ( $2n=60$ )  $\times$  self

The chromosome numbers of the parents involved in these crosses have been reported previously (13, 14, 23, 7, 25). It is the purpose of this paper to report the chromosome numbers of such hybrids, emphasizing especially the contribution of the triploid or pentaploid parent.

<sup>1</sup> Contribution no. 494, Maryland Agricultural Experiment Station.

### Material and methods

All chromosome determinations were made from root tips or droppers. These tips were removed from seedlings or one- or two-year-old bulbs, which were produced as the result of crosses made in 1934 and 1935. Fixation was made in Allen and Wilson's modification of Bouin's fluid, and the material was then treated with the usual xylol-paraffin technique. Sections were cut 18–22  $\mu$  thick and stained in iron-alum haematoxylin. Observations were made with the aid of a 15 $\times$  compensating ocular and a 90 $\times$  1.3 apochromatic oil immersion objective.

In each of the following crosses, the chromosome number contributed by the triploid or pentaploid has been derived by subtracting the gametic number of the other parent from the chromosome number obtained in the root tips of the hybrid seedlings. Information from other crosses has demonstrated that the diploids and tetraploids produce the expected chromosome number in the gametes.

### Results

#### HYBRIDS WITH TRIPLOID PARENT

##### 1. *T. lanata* (3*n*) $\times$ *tubergeniana* (2*n*)

In the material collected from the plants of this cross it was possible to determine the chromosome number of 35 hybrids. The results (table 1) indicated that the triploid parent contributed 15 and 18 chromosomes to the hybrids most often, although the variation in the functional gametes may extend from 12 to 21.

TABLE 1

Chromosomes from triploid...	12	13	14	15	16	17	18	19	20	21	22
No. of plants.....	1	2	4	10	2	3	8	0	2	3	0

##### 2. Inglescombe Yellow (3*n*) $\times$ John Ruskin (2*n*)

This cross is similar, in so far as chromosome number of the parents is concerned, to the previous one. The results are somewhat comparable in that the triploid contributed 15 and 18 chromosomes most frequently, although those gametes with 13 chromosomes were effective. The variation was from 12 to 20 chromosomes in the 28 hybrids obtained (table 2).

TABLE 2

Chromosomes from triploid.	12	13	14	15	16	17	18	19	20
No. of plants.....	2	6	2	8	2	1	6	0	1

3. John Ruskin ( $2n$ ) $\times$ Inglescombe Yellow ( $3n$ )

When the triploid is used as the male parent of the hybrid, the results (table 3) are considerably different. Although the range of chromosome numbers is about as extensive, the hybrids with 25 chromosomes are predominant in the progeny, showing that the triploid contributed 13 chromosomes to the hybrids more often than any other number.

TABLE 3

Chromosomes from triploid...	12	13	13+	14	15	16	17	18
No. of plants.....	2	19	1*	6	1	6	2	1

\*Thirteen plus a fragment.

4. Clara Butt ( $2n$ ) $\times$ Inglescombe Yellow ( $3n$ )

From this cross only a few plants were obtained and the chromosome numbers of three of these were determined. One hybrid had 25 chromosomes, one had 25 plus a fragment, and the third had 26 chromosomes.

## HYBRIDS WITH PENTAPLOID PARENT

It is apparent that the progeny are few in those crosses in which one parent was a pentaploid. Such hybrids are difficult to obtain, and furthermore the few seeds are either non-viable or the seedling succumbs so that a bulblet never forms. The following results, however, indicate that it is possible to obtain viable seed from crosses involving the pentaploid as one parent and a diploid or tetraploid as the other. It was also possible to obtain progeny from a selfed pentaploid.

5. *T. clusiana* ( $5n$ ) $\times$ *chrysantha* ( $4n$ )

Although only three seedlings from which chromosome counts could be obtained were available, the variation in the functional gametes of the pentaploid is apparent. In one the pentaploid contributed 21 chromosomes, in another 22, and in the third 29.

6. *T. chrysantha* ( $4n$ ) $\times$ *clusiana* ( $5n$ )

The results from the reciprocal of this cross (table 4) were determined from seven seedlings which survived. The hybrids ranged in

chromosome number from 49, just one more than the somatic number of the tetraploid, to 60, the pentaploid number. The pentaploid contributed 26 and 25 chromosomes respectively to the hybrids the greater number of times.

TABLE 4

Chromosomes from pent-

aploid.....	25	26	27	28	29	30	31	32	33	34	35	36
No. of plants.....	2	3	0	0	0	1	0	0	0	0	0	1

7. *T. clusiana* (5n) × *stellata* (2n)

Seven seedlings from this pentaploid-diploid cross showed the usual variation in their chromosome number (table 5). There is a slight predominance of 37 and 40 chromosome hybrids, indicating that the pentaploid as the female parent contributed 25 and 28 chromosomes respectively the greater number of times. However, the chromosome numbers of all the progeny range from 22 to 28.

TABLE 5

Chromosomes from pentaploid..	22	23	24	25	26	27	28
No. of plants.....	1	0	1	2	1	0	2

8. *T. clusiana* (5n) × self

Since there is no means of determining how many chromosomes the male and the female each contribute to the hybrid, table 6 is presented to show the chromosome numbers of the progeny.

TABLE 6

Chromosome number of hybrids....	47	48	49	50	51	52	53	54	55	56	57	58	59	60
No. of plants	1	2	0	1	0	0	1	1	0	0	1	0	0	1

### Discussion

A great many studies concerning meiosis in triploids and pentaploids have indicated that the reason for sterility in these types was due to the irregular gamete formation. Moreover it has been assumed that the chromosome distribution during the divisions showed what the gametes would have.

Practically equal division of chromosomes to the gametes, or the number representing one-half the somatic number, has been found by the investigators in the following genera: *Impatiens* (19), *Zea*

(11), *Lycopersicum* (9), *Petunia* (20), *Oenothera* (3), *Triticum* (22), *Hyacinthus* (1), *Datura* (2), and *Lilium* (18).

NEWTON and DARLINGTON (13), working with triploid and pentaploid *Tulipa*, and CHANDLER, PORTERFIELD, and STOUT (4) in a recent paper on the meiosis of triploid *Lilium*, found numerous irregularities in the prophase of the first division, so that lagging and eventual elimination of chromosomes often occurred. As a result, gametes would be formed which contained a smaller number of chromosomes than if this elimination had not taken place.

Recently SATINA and BLAKESLEE (15, 16) examined meiotic divisions of both male and female gametophytes in triploid *Datura* and found practically equal chromosome distribution in the first division, but subsequent irregularities reduced the population of these gametes and increased the population with unevenly divided or lower chromosome numbers. WOODS (24) in *Tulipa* observed the nearly equal distribution of chromosomes in the gametes at the first division, but some irregularities were present in later divisions so that the resulting gametes contained aneuploid numbers.

DARLINGTON (5) has stated: "The zygote progeny (however) do not correspond at all with the gametic proportions." The results of this immediate study indicate that the triploid, serving either as the male or female parent, rarely contributes the 18 chromosomes expected as a result of equal chromosome-carrying gametes. On the contrary, it is obvious that the functional gametes of the triploid carry considerably less than 18 chromosomes. When used as a female parent, in three different crosses, it contributed 15 chromosomes most frequently. As the male parent, 13 and 14 were the predominating number of the functional gametes.

This behavior of the triploid gametes of *Tulipa* is not unusual in the light of similar work now in the literature. There is evidently an elimination of those gametes carrying the higher chromosome number, so that the functional gametes contain numbers equaling or closely approximating the diploid number. As WOODS (24) has pointed out in a previous paper, bridging and fragmentation are common in the meiotic division of a triploid *Tulipa*. This probably accounts in part for the eventual elimination of chromosomes in the gametes of these triploids.

HALL (7) mentions similar results in some triploid *Tulipa*. He obtained aneuploid seedlings where the triploid has contributed from 12 to 22 chromosomes. He does not discuss the parents nor the way in which the cross was made.

DERMEN (6) crossed diploid *Malus* on triploid and obtained aneuploid hybrids ranging from the diploid to the triploid chromosome number. LEVAN (10), working with *Allium*, made reciprocal crosses between diploids and triploids and found that "the elimination or non-formation of aneuploid zygotes was greater when the embryo developed on a diploid than on a triploid." When the diploid served as the female parent, 53 of the 71 hybrids had the diploid number. When the triploid served as the female parent, aneuploid hybrids ranging from the diploid to the triploid number were obtained. MCCLINTOCK (12), working with *Zea*, found that in the triploid there was a "decided selection against extra chromosome-carrying male gametes and a less obvious selection against extra chromosome-carrying eggs." When the triploid served as seed parent, this cross indicated that not only gametes with the diploid number (no extra chromosomes) were functional, but also those with one and two extra chromosomes in excess of the diploid number. STOUT (21) made reciprocal crosses between diploid and triploid species of *Hemerocallis* and found that the triploid contributed the same number as the diploid most frequently to the hybrids, but the aneuploid numbers ranging up to twice this also functioned.

SATINA, BLAKESLEE, and AVERY (17) studied the chromosome number in the seed obtained from a triploid  $\times$  diploid cross and found that there were 816 times the number of diploid individuals expected on the basis of random assortment of chromosomes. LAMMERTS (8), in a diploid  $\times$  triploid cross of *Nicotiana*, found that the triploid gametes with the expected chromosome number, assuming equal distribution of chromosomes in the gametes, functioned most frequently.

According to HALL (7), "the pentaploid *T. clusiana*, though grown in quantity for commerce, is generally regarded as sterile, as might be expected from its pentaploid constitution." He states, however, that seedlings have been obtained on one or two occasions, but the parentage is unknown and occasionally this species set seed spontaneously. The seedlings had the pentaploid chromosome num-

ber, while those which appeared spontaneously were aneuploids with 50-52 chromosomes.

In the crosses involving *T. clusiana*, the pentaploid, variations in the hybrid seedlings are indicative of the irregular gamete formation in pentaploids. NEWTON and DARLINGTON (14) pointed out that this species forms a large proportion of aneuploid gametes. From observations of the first meiotic division of the pollen mother cells, they found associations at metaphase including quinquevalents, quadrivalents, trivalents, bivalents, and as many as ten univalents. The random distribution of the 60 chromosomes to the respective daughter cells would necessarily result in irregular numbers in many of the gametes, and confirmation of this is found in the results of the four *clusiana* crosses.

In the first of these, *T. clusiana* × *chrysantha*, assuming that the tetraploid male gametes form normal gametes of 24 chromosomes as in preliminary reciprocal crosses with a diploid, the pentaploid has formed functional gametes of 21, 22, and 29 chromosomes. In the second cross, *T. chrysantha* × *clusiana*, the pentaploid pollen showed great variation in chromosome number, for the range in the functional gametes extended from 25 to 36, although the frequency is highest in the neighborhood of 25 and 26.

The *T. clusiana* × *stellata* cross showed less variation in the chromosome number of the functional gametes, the range extending from 22 to 28, and no outstanding high frequency for any particular one of these.

The most unusual fact concerning the last of these crosses, *T. clusiana* × self, is that it was actually possible to obtain viable seed that survived. The ranges in the chromosome numbers of the functional gametes are hardly enlightening, except to show that there is great variation. Obviously there are no means of determining how many chromosomes the male or the female contributed.

From a consideration of these four crosses involving the pentaploid *T. clusiana*, it will be seen, as in the case of the triploid, that the pentaploid might form gametes containing one-half the somatic chromosome number, but they do not function as often as those gametes containing less than the expected number. From the work of NEWTON and DARLINGTON (14) previously mentioned, however,

one does not anticipate the formation of such equal chromosome-bearing gametes. Rather, as in the triploid, there has probably been an elimination of chromosomes so that the functional gametes carry less than the expected number.

### Summary

1. Seedlings have been obtained from reciprocal crosses involving both triploid and pentaploid forms of *Tulipa*. The other parents of these crosses were diploids and tetraploids.
2. The chromosome numbers of these progeny have been determined. The majority of them are aneuploids.
3. The triploid, either as a male or female parent, tends to contribute less than one-half the somatic number of chromosomes. This is probably explained by the irregularities in the meiotic divisions of this type.
4. Although the number of progeny from the pentaploid is small, a similar condition exists.

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# FURTHER EXPERIMENTS ON THE RELATION OF VITAMIN B<sub>1</sub> TO THE GROWTH OF GREEN PLANTS<sup>1</sup>

JAMES BONNER AND JESSE GREENE

(WITH ONE FIGURE)

## Introduction

In an earlier paper it has been shown (5) that some plants grown in sand culture in the greenhouse may respond with marked increases of growth to the addition of minute amounts of vitamin B<sub>1</sub> in the nutrient solution. The present paper reports further data relative to the vitamin B<sub>1</sub> economy of the green plant.

**MATERIAL AND METHODS.**—Plants were grown in quartz sand washed free of organic matter and contained in 2-gallon glazed crocks supplied with drainage. Shive's R<sub>2</sub>S<sub>3</sub> nutrient containing the minor elements was used for the majority of the experiments. The nutrient solution was made up from chemically pure reagents in order to avoid contamination by organic matter as far as possible. The nutrient was supplied at the rate of 250–500 cc. per crock on alternate days, the amount depending on the size of the plants and on weather conditions. On intervening days the crocks were supplied with tap water. In experiments where vitamin B<sub>1</sub> was to be used, it was added at the rate of 0.01 mg. per liter of nutrient solution unless otherwise noted.

A number of the experiments were conducted during the winter of 1938–1939. The light conditions prevailing during this time permitted vigorous growth in the greenhouse.

The "long photoperiod" conditions referred to here consisted of daily light periods varying from 18 to 20 hours. The natural day was supplemented with light from Mazda lamps which gave an intensity of approximately 75 foot-candles at the leaf surface. Conditions of "short photoperiod" refer to a daily light period of 9 hours.

<sup>1</sup> Work carried out with the aid of the Works Progress Administration, Official Project no. 665-07-3-83, Work Project L-9809.

Vitamin B<sub>1</sub> determinations were carried out by means of the *Phycomyces* assay (8, 4). Samples of the leaves to be analyzed were collected, generally between 11 A.M. and 2 P.M., dried at 60° C. for 48 hours, ground, and the vitamin determined in triplicate 10-mg. samples. The values obtained with the assay in this manner were found to be in good agreement with those obtained on the same material by the thiachrome fluorescence method after extraction, adsorption on Fuller's earth, and elution of the vitamin.<sup>2</sup> Because of its relative ease and simplicity, the *Phycomyces* assay was used in preference to the fluorescence method. It seems unlikely that sub-

TABLE 1

PHYCOMYCES ASSAY FOR VITAMIN B<sub>1</sub> IN THE PRESENCE OF LEAF TISSUE

CONTENTS OF TEST FLASKS (IN ADDITION TO 10 CC. PHYCOMYCES NUTRIENT)	VITAMIN B <sub>1</sub> (GAMMA)
Nothing.....	0.00
0.10 gamma vitamin B <sub>1</sub> .....	0.10
10 mg. ground leaf tissue ( <i>Schinus molle</i> )....	0.048
10 mg. ground leaf tissue+0.10 γ vitamin B <sub>1</sub> .....	0.148
	0.100 100% recovered
10 mg. ground leaf tissue ( <i>Eucalyptus</i> ).....	0.054
10 mg. ground leaf tissue+0.10 γ vitamin B <sub>1</sub> .....	0.156
	0.102 102% recovered

stances normally present in leaf tissue interfere in any way with the *Phycomyces* assay, since added vitamin can be recovered quantitatively in the presence of such tissue (table 1).

## Investigation

INFLUENCE OF VITAMIN B<sub>1</sub> ON ACCUMULATION OF DRY WEIGHT

The rate of accumulation of dry weight, both of root and of shoot, appears to be increased under the influence of added vitamin B<sub>1</sub> (table 2); and with certain species such as mustard, this increase may amount to several hundred per cent. Results similar to those presented in table 2 have been obtained with numerous other species of plants, for example *Agrostis tenuis* and *Poa trivialis*. These were

<sup>2</sup> We are indebted to ERICK HEEGARD for the thiachrome determinations, and to E. WADDELL for the execution of the *Phycomyces* assays.

grown in sand under conditions similar to those just outlined and the grass was clipped periodically. In 2 months the cultures of *P. trivialis* which received vitamin B<sub>1</sub> produced a total of more than 13 gm. dry weight of clippings per crock whereas the control crocks produced but 2.05 gm. *Agrostis tenuis* which received vitamin B<sub>1</sub> produced during the same time more than four times as great a dry weight of clippings as the controls.

Plants grown in soil may be induced to accelerate their rate of dry weight deposition by additions of vitamin B<sub>1</sub>, as shown by the fol-

TABLE 2  
RELATION OF ADDED VITAMIN B<sub>1</sub> TO ACCUMULATION  
OF PLANT DRY WEIGHT

SPECIES	LENGTH OF EXPERI- MENT (WEEKS)	NO. PLANTS	DRY WEIGHT (GM. PER PLANT)					
			TOPS		PER- CENT- AGE IN- CREASE	ROOTS		PER- CENT- AGE IN- CREASE
			CON- TROL	B <sub>1</sub>		CON- TROL	B <sub>1</sub>	
<i>Xanthium pennsylvanicum</i> (short photoperiod)	7	80	0.72	1.15	160	0.061	0.12	197
<i>Brassica alba</i> (short photoperiod).....	6	40	0.064	0.29	453	.....	.....	.....
<i>Brassica nigra</i> (short photoperiod).....	6	40	0.047	0.175	372	.....	.....	.....
<i>Cosmos</i> (long photoperiod)	8	50	0.0675	0.110	163	0.0134	0.0183	137

lowing experiment. *Cosmos* seedlings were planted in 4-inch pots in an open garden soil and maintained in the greenhouse. The experimental plants were watered with a solution containing 0.1 mg. of vitamin B<sub>1</sub> per liter while the controls received tap water. After 6 weeks the plants supplied with the vitamin were found to have an average dry weight per shoot (stem and leaves) of 0.33 gm. while the control plants had an average of 0.080 gm. per shoot, or less than one-fourth that of the treated plants.

In all these cases, as well as in many others, the plants which received the vitamin exhibited a greater general vigor than the control plants, as indicated by taller shoots, larger leaves, and more extensive root system. On the basis of the results presented in table 2,

it seems justifiable to conclude that this increase in vegetative vigor is accompanied by an actual increase in accumulation of dry matter.

#### EFFECTIVE CONCENTRATIONS OF VITAMIN B<sub>1</sub>

Table 3 gives the results of an experiment in which *Cosmos* seedlings, grown in sand culture, were supplied with nutrient solution containing varying amounts of vitamin B<sub>1</sub>. The plants were

TABLE 3

RELATION OF VITAMIN B<sub>1</sub> CONCENTRATION OF NUTRIENT SOLUTION TO DRY WEIGHT DEPOSITION BY COSMOS SEEDLINGS. PLANTS MAINTAINED UNDER CONDITIONS OF LONG PHOTOPERIOD

CONCENTRATION OF NUTRIENT SOLUTION	DRY WEIGHT (GM. PER SHOOT)	PERCENTAGE IN- CREASE OVER CONTROL
None (nutrient only)..... Mg. per liter	0.058	.....
0.1.....	0.105	81
0.01.....	0.138	138
0.001.....	0.111	91
0.0001.....	0.075	29

harvested 4 weeks after the appearance of the first leaves, and dry weights determined. The optimum concentration of vitamin B<sub>1</sub> for the promotion of dry weight deposition appears to be 0.01 mg. per liter of nutrient solution under these conditions, but a detectable effect was exerted by nutrient solution containing only 0.0001 mg. or 0.17 of vitamin B<sub>1</sub> per liter.

#### EFFECTS OF VITAMIN B<sub>1</sub> OVER EXTENDED PERIODS

In an earlier paper (5), as well as in the experiments of table 2, the plants were harvested and the results obtained after periods of 4-10 weeks. In figure 1 are presented the growth curves, as measured by total height of shoot, of two sets of *Ceratonia siliqua* (carob tree) seedlings maintained in sand culture for 1 year. Although the control plants grew vigorously, the plants which received vitamin B<sub>1</sub> grew still more vigorously throughout the experimental period. After 1 year, the plants which received the vitamin continued to

grow at a faster rate than the controls. Similar although less striking results were obtained with seedlings of *Prunus ilicifolia* which were grown in soil in the open during a period of 1 year. Growth measurements made in experiments which extended through several months

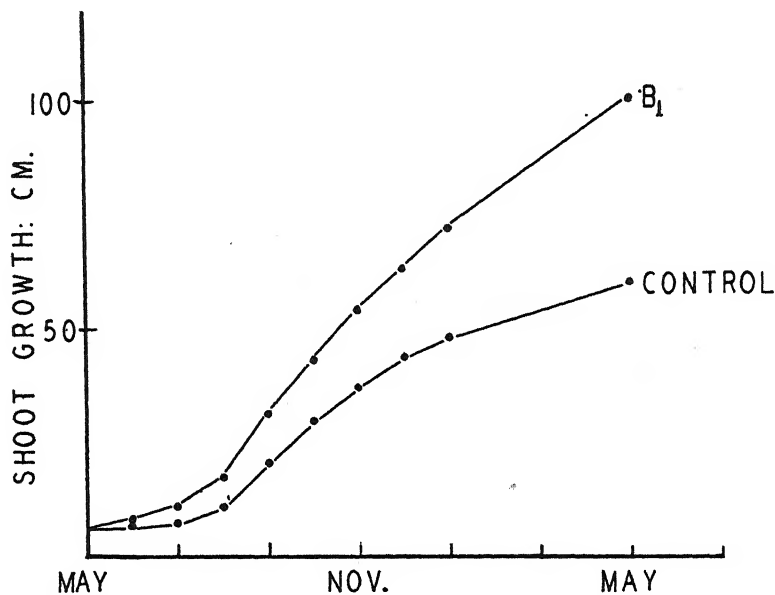


FIG. 1.—Growth of seedlings of *Ceratonia siliqua* in sand culture with and without addition of vitamin B<sub>1</sub> (0.01 mg. per liter).

are given in table 4. In every case the treated plants continued to grow more rapidly than the controls throughout the course of the experiment. Additions of vitamin B<sub>1</sub> apparently bring about a lasting rather than a temporary promotive effect on plant growth.

#### RELATION OF VITAMIN B<sub>1</sub> CONTENT TO PLANT RESPONSE

It has been pointed out (5) that certain species do not respond with increased growth to additions of the vitamin. Among such species are corn, wheat, pea, garden bean, and tomato. It is known both for pea (1, 3) and for tomato (7) that vitamin B<sub>1</sub> is indispensable for the growth of isolated roots, thus presumably it is indispensable for root growth in intact plants of these species.

In table 5 are tabulated vitamin B<sub>1</sub> contents of the leaves of several species of plants.<sup>3</sup> These leaves were taken from the control plants of experiments in which the growth response to added vitamin was also determined. The species found to respond to additions of the vitamin with increased growth possessed leaf vitamin contents between 0.0 and 6.0 mg. per kg. dry weight of leaves. The vitamin content of tomato leaves was found to be 18 mg. per kg., or at least three times as great as that of the species responsive to vitamin B<sub>1</sub>.

TABLE 4  
RESPONSE OF SEEDLINGS TO ADDED VITAMIN B<sub>1</sub>

SPECIES	LENGTH OF EXPERIMENT	No. PLANTS	GROWTH MEDIUM	GIVEN B <sub>1</sub>	NEW SHOOT GROWTH AT END OF EXPERIMENT (CM.)		RATIO OF B <sub>1</sub> /CONTROL (%)
					B <sub>1</sub>	CON-TROL	
<i>Cerantonia siliqua</i> ....	1 year	8	Sand	Alternate days	93	54	172
<i>Myrsine africana</i> ....	5 months	24	Sand	Alternate days	34	20	170
<i>Daphne odora</i> .....	6 months	8	Sand	Alternate days	3.6	1.1	327
<i>Correa ventricosa</i> ...	6 months	8	Sand	Alternate days	36	32	112
<i>Camellia japonica</i> ...	6 months	16	Sand	Alternate days	23	6.4	360
<i>Prunus ilicifolia</i> ....	1 year	12	Soil*	Weekly	233	178	131
<i>Cedrus libani</i> .....	5 months	16	Soil*	Weekly	42	17	247

\* Grown outdoors.

The leaves of pea plants contain two or more times as much vitamin as the leaves of responsive species.

It has been demonstrated in the case of the pea plant that vitamin B<sub>1</sub> is formed in the green leaves under the influence of light (5), and that in the normal plant the vitamin B<sub>1</sub> required by the root is supplied presumably by these leaves. Species with relatively high contents of leaf vitamin B<sub>1</sub> may not be limited in their growth by the available amounts of this substance. Those species of table 5 with relatively low (less than 6.0 mg. per kg.) content of leaf vitamin

<sup>3</sup> The determinations summarized in table 5 represent the vitamin contents of the leaves of the individual species under the conditions of the particular experiment. It has been found, as would be expected, that the vitamin content of leaves varies with external factors. This will be the subject of a later report.

appear to be limited in their growth by the available vitamin B<sub>1</sub>. The response of these latter species to added vitamin B<sub>1</sub> is not completely correlated with leaf vitamin content. Thus *Poa trivialis* with 4.2 mg. per kg. dry weight of leaves responds more strikingly than does *Agrostis tenuis* with 6.0 mg. of vitamin per kg. dry weight of leaves. On the other hand, *Brassica alba* responds to added vitamin somewhat more than does *B. nigra*, although the leaf vitamin content of the former is higher than that of the latter. That leaf vitamin B<sub>1</sub> content should not be completely correlated with response to

TABLE 5  
B<sub>1</sub> CONTENT OF LEAVES OF VARIOUS SPECIES OF PLANTS  
GROWN IN SAND CULTURE

PLANT	CONDITIONS	B <sub>1</sub> CONTENT OF LEAVES MG./KG. DRY WEIGHT	RESPONSE TO ADDED VITAMIN
Tomato.....		18.0	None
Pea.....		13.0	None
Brassica alba.....	Short photoperiod	4.9	+
Brassica nigra.....	Short photoperiod	3.0	+
Agrostis tenuis.....		6.0	+
Poa trivialis.....		4.2	+
Cosmos (autumn).....	Long photoperiod	5.0	+
Xanthium pennsylvanicum....	Short photoperiod	3.4	+
Ceratonia siliqua.....		3.0	+
Cattleya (hybrid).....		1.8	+
Camellia japonica.....		0.0	+

added vitamin would be in accordance with the view that although available vitamin B<sub>1</sub> may be a factor limiting the growth of these species, there may be still other growth factors which are to different degrees also limiting in the several cases. Leaves of *Camellia japonica* were found to contain no detectable amount of the vitamin. When vitamin B<sub>1</sub> was added to the nutrient solution, plants of this species were found to thrive in sand culture and to approximate in luxuriance plants grown in rich soil. Control plants which received no vitamin were found to grow very poorly in sand culture. It would seem possible that *Camellia* plants depend for their supply of vitamin B<sub>1</sub> largely on the rich soil with which they are supplied in usual horticultural practice.

RELATION OF VITAMIN B<sub>1</sub> NUTRITION TO VITAMIN CONTENT  
OF THE LEAF

When vitamin B<sub>1</sub> is supplied to the roots of plants grown in sand culture there is an increase in the amount of vitamin present in the leaves of these plants (table 6). It has not yet been possible to decide

TABLE 6  
ACCUMULATION OF VITAMIN B<sub>1</sub> IN LEAVES OF PLANTS  
GROWN IN SAND CULTURE WITH ADDED VITAMIN

SPECIES	VITAMIN CONTENT OF LEAVES (MG./KG. DRY WEIGHT)	
	SUPPLIED B <sub>1</sub>	CONTROL
<i>Brassica alba</i> (long photo-period).....	15.8	6.0
<i>Brassica nigra</i> (long photo-period).....	6.4	3.9
<i>Agrostis tenuis</i> .....	8.6	5.8
<i>Poa trivialis</i> .....	7.2	4.4
<i>Cosmos</i> (long photoperiod).....	6.0	5.0

whether this increase is to be attributed solely to passive uptake of the vitamin or to an increased synthesis of the vitamin by the treated plants.

### Discussion

Although the addition of vitamin B<sub>1</sub> exerts a promotive effect on the vegetative growth of many species of green plants, it has not yet been found to exert any specific effect on the initiation of the flowering state (6). In the present experiments, and also in others not reported here, plants have been maintained in culture until the flowering state was attained. In no case was an effect on the time of flowering or on the number or size of flowers or fruits observed, other than such as might be expected from the greater vegetative vigor of the treated plants. In no case have additions of vitamin B<sub>1</sub> been found to be in any way detrimental to flower production in the present experiments.

It has been shown (2) that the growth response of etiolated ex-

cised pea embryos of different strains to added ascorbic acid is a direct function of the quantity of the acid which each particular strain is capable of synthesizing for itself. It would appear that the response of different species of green plants to additions of vitamin B<sub>1</sub> similarly depends on the amount of this growth factor which each species is able to synthesize under the prevailing environmental conditions.

### Summary

1. Species of plants grown in sand culture under greenhouse conditions respond to addition of vitamin B<sub>1</sub> to the nutrient solution (0.01 mg. per liter) with marked increases in rate of dry weight accumulation. A similar influence of added vitamin on dry weight accumulation of plants grown in soil is also demonstrated.

2. Carob trees supplied on alternate days with vitamin B<sub>1</sub> in sand culture during 1 year continued to exhibit a greater growth rate than controls which did not receive the vitamin. This and other experiments indicate that the promotive effect of added vitamin is lasting rather than temporary.

3. Tomato, which does not respond to addition of vitamin B<sub>1</sub> with added growth, was found to contain at least three times as much vitamin in its leaves as was found in the leaves of other plants (*Cosmos*, *Poa*, *Brassica*, etc.) which do respond to such addition with added growth. *Pisum sativum*, another species unresponsive to additions of the vitamin, was found to contain more than twice as high a concentration of leaf vitamin as the responsive species. It is suggested that the amount of vitamin B<sub>1</sub> synthesized by the leaves of a given species regulates, at least in part, the response of that species to additions of the vitamin. It is possible that leaf vitamin content might be used to diagnose whether or not addition of vitamin B<sub>1</sub> would promote the growth of a given crop or species of plant.

4. When vitamin B<sub>1</sub> is supplied to the roots of plants grown in sand culture, the vitamin content of the leaves is increased over that of control plants not supplied with the vitamin.

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## COLOR AND KEEPING QUALITIES OF CUT FLOWERS

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 508

MARSHALL S. NEFF

Roses, such as the Hollywood variety, as well as many other red or pink flowers, take on a faint bluish cast under some storage conditions. Similarly flowers which have been held in storage at times do not last as long at ordinary room temperatures as do fresh flowers. Preliminary experiments indicate that these difficulties may be obviated by means readily available to those who are concerned with the production, storage, and marketing of cut flowers.

In the first experiment freshly cut Hollywood rosebuds were stored at 35°-40° F. in a 70-liter metal container while in a refrigerated chamber. Water to a depth of  $\frac{1}{4}$  inch in the bottom of the container and a sheet of window glass over the top, in place of the metal lid, maintained a high humidity within the container. The rosebuds were divided into three groups previous to being placed in the container. In group 1 the lower portions of the stems were immersed in a flask containing a commercially available solution designed to maintain flowers in a fresh condition over a considerable length of time provided the flower stems remain in it. Two and one-half times the amount of sugar recommended by the manufacturers was added to the solution. In group 2 the flower stems were kept dry, an empty flask being placed over their ends to keep the stems out of the water which covered the bottom of the container. In group 3 the lower portions of the stems were placed in the commercial solution (with the added sugar as noted) for an hour; they were then removed from the liquid and the bases of the stems covered with a dry empty flask as in group 2, and also placed in the container. All lots were illuminated, as soon as the groups were in place, with light from a 160 watt Mazda lamp set in a small reflector and suspended 8 inches above the glass of the metal container in the cold room. The light was first passed through a filter consisting of water to which had been added sufficient copper sulphate to produce a

light blue color. The flowers received approximately 50 foot-candles of light over a 168-hour period. During the last 17 hours of storage the copper sulphate solution was removed and the light intensity reduced so that the flowers received approximately 13 foot-candles. At the end of the storage period the color of the flowers was still fresh and lively, perhaps slightly less intense than in fresh flowers, but there was no bluing tendency. The flowers were removed from

TABLE 1

EFFECT OF STORAGE CONDITIONS UPON SUBSEQUENT KEEPING QUALITIES OF HOLLYWOOD ROSES AT ROOM TEMPERATURE; STORAGE PERIOD 185 HOURS AT 35°-40° F.

GROUP	HUMIDITY	HOURS WITH STEMS IN SOLUTION*	HOURS STORED DRY	APPROXIMATELY 50 FOOT-CANDLES (FILTERED)†	APPROXIMATELY 13 FOOT-CANDLES (UNFILTERED)	NUMBER OF TURGID FLOWERS AT ROOM TEMPERATURE. FOUR FLOWERS USED IN EACH GROUP. STEMS IN WATER					
						1 DAY	4 DAYS	5 DAYS	6 DAYS	8 DAYS	9 DAYS
1.....	High	185	0	168	17	4	4	4	4	1	1
2.....	High	0	185	168	17	4	4	4	1	1	.....
3.....	High	1	184	168	17	4	4	2	.....	.....	.....
4 Fresh roses (check).....	.....	.....	.....	.....	.....	4	4	1	.....	.....	.....

\* A commercial product mixed with water and sugar.

† Light filtered through a layer of water to which was added sufficient copper sulphate to produce a light blue color.

storage and taken to the laboratory where the temperature was approximately 70° F. They were placed with the lower portions of their stems in water in order to compare their keeping qualities with freshly cut buds also held at room temperature. The flowers stored dry tended to droop during the storage period, but they revived at room temperature when their stems were in water (table 1).

The table indicates that roses which have been stored may last longer in water at room temperature than fresh roses under the same conditions. The one-hour period of immersion of the stems in the commercial solution and subsequent dry storage proved less effective in maintaining good color and a fresh condition than when the stems were not placed in the commercial solution first. On the

other hand, maintenance of the stems in the solution during the entire storage period gave the best results as to color and keeping qualities. By the sixth day at room temperature the fresh roses (group 4) were a dull bluish color and the petals were wilted and dried. Treated roses (group 1) were turgid and retained a lively pink color. The roses of group 2 retained a brighter color and remained fresh longer than fresh flowers which had not been stored.

In a second experiment roses were held one week in a sealed metal container under conditions of 34°–38° F., high humidity, darkness, and the stems out of water or solution of any kind. During the week in darkness the petals lost the characteristic glow of fresh flowers and there was a tendency to become blue. At the end of the week the flowers were removed from the container but kept in the cold room at the same temperature as previously. The stems of these flowers were then placed in a solution of the following composition: 1000 cc. water; 100 gm. glucose or sucrose; 0.5 gm. potassium nitrate. With the bases of the stems in this solution they were then returned to the container. Window glass was again substituted for the metal cover so that it was possible to expose the flowers for 185 hours to the same conditions of light, temperature, and humidity as detailed in the first experiment. During this treatment the bluish color which developed during the storage period in darkness was eliminated. The roses remained turgid after they were removed to room temperature and the stems placed in water. They retained a lively color for a considerable time. No comparison was made with fresh flowers in this case.

The commercial solution used in the first experiment was also tested in conjunction with low temperature, low humidity, and direct Mazda light. Freshly cut rosebuds were placed in the 34°–38° F. room with the lower portions of their stems in a jar containing the commercial solution. A beaker full of dry calcium chloride was placed next to the flowers and a bell jar inverted over both the flowers and the calcium chloride. A short piece of glass tubing running through a rubber stopper in the top of the bell jar permitted inward and outward diffusion of gases. The exact humidity maintained was not determined but the lack of any accumulation of water droplets on the sides of the bell jar indicated a much lower humidity than previously used. No light filter was used. The flowers received direct Mazda light for 185 hours. When removed from the ball jar

to an open room at 70° F. and the stems placed in water the treated flowers had better keeping qualities than fresh roses. In addition, the color was very similar to that of group 1 in the first experiment. In this latter experiment the foliage was bleached somewhat and tended to wither and dry more rapidly than in the untreated specimens.

In another experiment freshly cut rosebuds were divided into two lots. The stems of one group were placed in the potassium nitrate-sugar solution, already mentioned, and the stems of another in tap water. No metal container or bell jar was used. The flowers were placed in a room at 70° F. Suspended in the room was an arc light, screened in with glass to eliminate any injurious ultra-violet rays. Approximately 800 foot-candles of light reached the flowers. They were maintained in the light for about 18 hours. At the end of this period the color was still bright and lively but the intensity was decreased slightly. When these treated flowers were removed to an open room at 70° F. and their stems placed in water under usual conditions, the color of those which had the lower portions of the stems in the potassium nitrate-sugar solution during the 18 hours of light treatment remained lively and fresh until the petals wilted. They showed much less tendency to turn blue than did untreated roses. Roses that had been in water only, during the light treatment, had less tendency to turn blue than those without any light treatment, but their petals began to take on a bluish cast with maturity. The treated roses of either type lasted longer than fresh flowers when held at room temperature.

Further investigations are now in progress to determine the optimum conditions for maintaining color and keeping qualities of flowers. It is obvious that light, either from a screened arc lamp, direct from a Mazda lamp, or filtered through a copper sulphate solution, is of great aid in extending the life of cut flowers in water at room temperature and preventing the tendency of red flowers to turn blue. This is particularly true when used in association with dry storage, or when the stems are immersed in appropriate solutions.

## LAYERING IN EASTERN WHITE PINE

H. J. LUTZ

(WITH ONE FIGURE)

Natural layering appears to be exceedingly rare in *Pinus*. MAYR (2) stated that it occurs in the group but did not mention specific examples. In his discussion of reproduction by layering among conifers, COOPER (1) cited MAYR and also mentioned a possible example of layering in *Pinus montana* described by SCHRÖTER. Apparently natural layering in *Pinus* has not previously been reported in America. The example here reported was observed in the Yale Demonstration and Research Forest, near Keene, New Hampshire, during the summer of 1938.

The parent tree, a white pine, originated about 30 years ago at the foot of a short slope in an old field. The soil was Merrimac coarse sand. Ground vegetation was sparse and the pine trees were scattered so that considerable erosion occurred on the slope. Eroded material accumulated to a depth of 15-25 cm. around the tree, covering portions of the lowest whorl of branches with a mixture of mineral soil material and organic debris. One of the basal branches on the south side of the tree developed roots. The parent tree and the rooted basal branch, which had assumed an upright habit, are illustrated in figure 1. The rooted branch was about 2.4 m. tall, and at the point of attachment to the bole was 4.4 cm. in diameter. The roots were traced for a horizontal distance of 1.8 m. but their ends were not reached. The parent tree was 15.2 cm. in diameter.

It is well known that layering frequently occurs in various gymnosperms, for example, *Tsuga*, *Picea*, *Abies*, *Chamaecyparis*, *Thuja*, *Juniperus*, and *Taxus*. The rare occurrence of the phenomenon in *Pinus* can scarcely be explained on the basis of unfavorable habitat conditions since pines commonly occur in association with other gymnosperms which do layer. It would seem that the intolerance of most pines to shade may be an important consideration in this con-



FIG. 1.—Basal branch of *Pinus strobus* which has developed roots and assumed an upright habit.

nection. Because of intolerance, the basal branches of most pines may die before sufficient organic debris has accumulated to cover them partially and thereby create conditions favorable for rooting.

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1. COOPER, WM. S., Reproduction by layering among conifers. BOT. GAZ. 52: 369-379. 1911.
2. MAYR, HEINRICH, Waldbau auf naturgesetzlicher Grundlage. Berlin. 1925.

## CURRENT LITERATURE

*Hundert Jahre Zellforschung.* By L. ASCHOFF, E. KÜSTER, and W. J. SCHMIDT. Protoplasma-Monographien, Band 17. Berlin: Gebrüder Borntraeger, 1938. Pp. x+285.

This volume includes three of the many discussions of the cell theory, its history and development, whose appearance has marked the close of a century since SCHLEIDEN and SCHWANN announced the universal significance of cellular structure. KÜSTER treats the development of the theory of the plant cell (62 pages); SCHMIDT discusses the development of the theory of the animal cell and its present position and significance (101 pages); and ASCHOFF deals with VIRCHOW's cellular pathology (99 pages).

The space available has allowed only a summary review of the results of innumerable workers. In consequence, each paper has become somewhat of an essay presenting the views of the writer rather than an adequate presentation of varied views upon controversial topics. This is particularly true in KÜSTER's short outline of the study of the plant cell. Both he and SCHMIDT have given individual credit to the earlier investigators, omitting almost entirely the citations of those of later years. ASCHOFF, dealing with a more restricted field, treats it somewhat more fully.

While the contributions of the three authors are distinct, there is evidence of collaboration. Little repetition appears, and there seems to be general agreement upon matters of opinion. Inevitably many of the judgments expressed will be unacceptable to some readers. An illustration of a ready source of controversy is the emphasis laid by KÜSTER upon the importance of SCHLEIDEN's contribution. Some other discussions that have appeared within the last two years have insisted more strongly upon the knowledge of plant cells available before the appearance of SCHLEIDEN's rather dogmatic little *Beiträge zur Phyto-genesis*. SCHMIDT seems to agree with KÜSTER's views on this point, although he necessarily devotes more attention to SCHWANN's study of the animal cell.

One result of the brevity and comparative lack of "documentation" in the volume is that it is easily readable. It will be of use to the non-cytologist who may wish a hurried view of an extensive field. It will not serve as a work of reference for one who wishes to dig below the surface.—C. E. ALLEN.

*Plant Physiology.* By BERNARD S. MEYER and DONALD B. ANDERSON. New York: D. Van Nostrand Co. 1939. Pp. 696. \$4.50.

During the last two years elementary textbooks of plant physiology have appeared so frequently that this branch of botany now seems well supplied with texts. There is always room, however, for a superior work, and this volume leaves little to be desired. It is well written, accurate, clear, and for the most

part logically arranged. The only feature of the organization that seems open to criticism occurs in the presentation of respiration, in which aerobic respiration, the last half of the process, is treated before anaerobic respiration, the first half. There is a common but fallacious notion that students are more familiar with aerobic than with anaerobic phases of respiration, and that the better known processes should be treated first—proceeding from the known to the unknown. As a matter of fact students are just as ignorant of aerobic processes as they are of anaerobic; and in presenting aerobic respiration first, one loses the continuity of the process. Moreover, all doubts of the continuous nature of anaerobic and aerobic processes have been swept away by recent investigations.

There are thirty-seven chapters, the earlier of which deal with such fundamentals as properties of solutions, interfacial phenomena, colloidal systems, properties of gels and sols, osmosis, imbibition, permeability, etc. Then the physiological processes are considered in logical order, followed by chapters that deal with the organism as a whole, growth, hormones, correlations, periodicity, and movements of adjustment.

There are 151 well-chosen text figures, and good bibliographies at the close of each chapter for supplementary reading. The work seems to present a satisfactory treatment of the subject as it is understood at the present time. Teachers and students should enjoy using a text that is so well balanced, so accurate and so well arranged.—C. A. SHULL.

*Moss Flora of North America North of Mexico*. Vol. I, Part 3. By A. J. GROUT. Newfane, Vermont: Published by the author, 1938. Pp. 137-192. Pls. 22. \$2.50.

This part includes the families Encalyptaceae, Buxbaumiaceae, and Pottiaceae. (For reviews of previous parts see BOT. GAZ. 88:111; 1929. 93:110; 1932. 96:578; 1935.) The first family is revised by SEVILLE FLOWERS, with a key to sterile specimens of *Encalypta* contributed by the editor. The genera *Pleurochaete* and *Tortella* (Pottiaceae) were contributed by INEZ M. HARING. WILLIAM C. STEERE furnished the revision for *Barbula* and *Didymodon*. All other genera have been revised by the editor. Especially noteworthy is the trend to synonymize species of genera commonly considered to be difficult taxonomically. In the revision of *Barbula* more than seventy species are reduced to eighteen, and one is described as new. Type specimens were studied in many instances. As in previously issued parts, the plates constitute nearly half the publication.—P. D. VOTH.

*Anatomie der Vegetationsorgane der Pteridophyten*. By Y. OGURA. Handbuch der Pflanzenanatomie. By K. LINSBAUER. II. Abt., Band VII, 2. Teil: Archegoniaten, B. Berlin: Gebrüder Borntraeger, 1938. Pp. viii+476. Illustrated. Rm. 60.

This volume is a summary of all published anatomical investigations on the vegetative organs of the Pteridophyta up to 1935. The general part is devoted

to the stem, leaf (including sporophyll and leaf apex), and root. All Pteridophyta are subdivided in the second part, where their anatomy is adequately discussed, into the Aphyllata (Psilophytean forms), the Microphyllata, and the Macrophyllata. Arrangement of smaller taxonomic groups within these subdivisions follows accepted modern interpretations. Although relatively few of the 382 figures are original, the illustrations are to the point and well chosen. More than 1200 literature citations are listed. There are indices for authors, for scientific names of plants, and for subjects. This book will serve as an excellent source for anatomical information.—P. D. VOTH.

*Applied Silviculture in the United States.* By R. H. WESTVELD. New York: John Wiley & Sons, 1939. Pp. vii+567. Figs. 102.

The entire United States is divided into eighteen regions characterized by their type of forest vegetation. Each is treated as a unit with reference to its physiographical and ecological features, past practices, and suggestions for future development, maintenance, and management. There are many good, pertinent illustrations and a fairly comprehensive bibliography at the close of each chapter.—E. J. KRAUS.

*The Leguminous Plants of Wisconsin. The Taxonomy, Ecology, and Distribution of the Leguminosae Growing in the State Without Cultivation.* By NORMAN C. FASSETT. Madison, Wisconsin: University of Wisconsin Press, 1939. Pp. xiii+157. Illustrated. \$3.00.

Ninety-seven species and varieties belonging to twenty-seven genera of Leguminosae have been reported. Individuals of these species were studied in the field as well as in numerous herbaria, so that this attractive publication is the result of years of study. The chief artificial keys are based on vegetative characters; additional keys are based on flower, fruit, and seed differences. Every species and variety is discussed, and there is a critical chapter on the epidermal outgrowths of the plants. Distribution maps are included. Excellent photographs, some showing the vestiture of leaves and stems, increase the value of the book.—P. D. VOTH.

*Keys to the Phyla of Organisms Including Keys to the Orders of the Plant Kingdom.* By FRED A. BARKLEY. Missoula, Montana: Associated Student's Store, 1939. Pp. iv+39. 75¢.

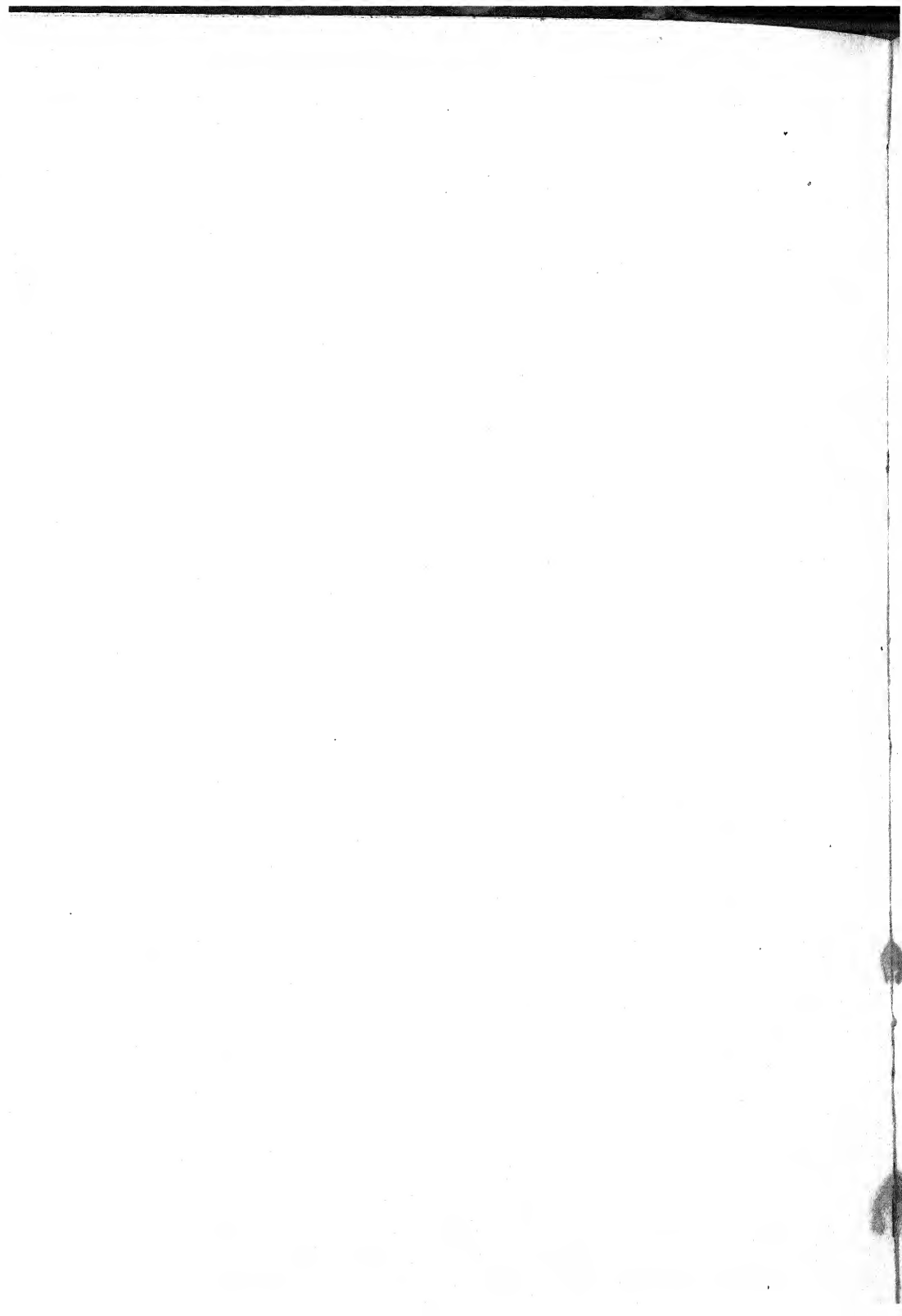
These keys are designed to aid in placing unknown non-nucleated organisms, the Protista (Euthallophyta and Protozoa), and all remaining plants into the right orders; and the remaining animals into the respective phyla. An outline of the classification used forms a concise list of the main divisions of living and fossil organisms and indicates that the author is in agreement with many of the recent publications on classification. The glossary is sufficiently detailed to aid in the use of the keys. A selected list of references is appended.—P. D. VOTH.

*Form- und Stoffwechsel der Golgi-Körper.* By G. CHR. HIRSCH. Protoplasma-Monographien, Band 18. Berlin: Gebrüder Borntraeger, 1939. Pp. xi+394.

The extensive literature dealing with Golgi material in animal cells is reviewed and criticized in the light of the author's conception of its history, nature, and function. HIRSCH's conclusions are briefly as follows:

While it is always possible to distinguish mitochondria from Golgi bodies, there is nevertheless an intimate relation; either products of mitochondria are transformed directly into Golgi material or materials or enzymes produced by mitochondria function in the organization of Golgi material. The latter appears first as a "pre-substance," usually as structureless, irregular or rounded bodies which may be aggregated into a network. From the substance in this metabolically inactive state arise the active "Golgi systems," lying either isolated or in a localized region of the cell. The often-described appearance of a network at this stage is, according to HIRSCH, an artifact due to the adsorption of osmium tetroxide or silver nitrate on the surfaces of closely aggregated but distinct bodies. Each system becomes differentiated into an external portion still staining deeply with osmium or silver and an internal portion which is stained more lightly if at all. From the internal portion develops the "product," a secreted substance characteristic of the particular type of cell concerned. The "product" may be any one of a variety of substances, including albumins, slime, pigments, oils, fats, and hormones. Whether the Golgi systems involved in the formation of so diverse products are to be considered as belonging to a single category is left an open question. Also uncertain is the fate of the fragments of the external portion of a Golgi system when the "product" is freed. It is possible that these fragments may participate in a second sequence of pre-substance, system, product. At least it seems clear that most commonly the history of a single cell includes several such sequences.

The still very scattered discussions of supposed Golgi bodies in plant cells are cited rather uncritically. Thus GUILLERMOND's identification of Golgi material with the plant vacuome, BOWEN's (tentative) identification with osmophilic platelets, and WEIER's identification with plastids are quoted, with no suggestion that these three conceptions are contradictory and mutually exclusive. An interesting item is the author's recognition (p. 262) of *Polytrichum* as a fern. The usefulness of the book is enhanced by extensive and easily usable tabulations of observations and conclusions regarding Golgi bodies (real or supposed), and by a full bibliography which is arranged first by topics (chiefly according to organs or tissues concerned), and second alphabetically under authors' names.—C. E. ALLEN.



# THE BOTANICAL GAZETTE

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## BIOCHEMICAL NITROGEN FIXATION STUDIES

### I. EVIDENCE FOR LIMITED OXYGEN SUPPLY WITHIN THE NODULE

FRANKLIN E. ALLISON, C. A. LUDWIG, SAM R. HOOVER  
AND F. W. MINOR<sup>1</sup>

(WITH SIX FIGURES)

#### Introduction

During the last four years various types of studies dealing with the metabolic changes occurring in legume nodules have been carried on at this laboratory. Some of the more interesting phases of the work already completed will now be reported in a short series of articles, of which this is the first. The primary purpose of all the work was to obtain fundamental information on the biochemical processes taking place in the nodule as a basis for a better understanding of the nitrogen fixing process occurring there. The information at present in the literature dealing with nodule metabolism is extremely meager.

The present paper presents evidence concerning the oxygen supply within the nodules. The general experimental methods used are also described briefly. Special techniques employed in certain phases of the work will be described in connection with the particular results concerned.

<sup>1</sup> The writers are indebted to ELLEN K. RIST of this laboratory for the nitrogen analyses and to E. C. BUTTERFIELD of the Bureau of Plant Industry for supplying much of the plant material.

### Methods

The general method used in most experiments was to culture detached nodules or roots for a few hours in a culture solution in a given atmosphere, and record the data desired. Usually the nodules were maintained in Warburg respiration vessels that were constantly shaken in a water bath at 28° C., and oxygen consumption or both oxygen consumption and carbon dioxide production measured as desired. In some cases, to be discussed in later papers, these data were supplemented by total gas analyses and special chemical determinations. Total nitrogen was determined by the usual Kjeldahl method.

The legume species used to the greatest extent in these investigations included: cowpea (*Vigna sinensis* (L.) Endl.), crown vetch (*Coronilla varia* L.), Korean lespedeza (*Lespedeza stipulacea* Maxim.), hairy vetch (*Vicia villosa* Roth), common vetch (*Vicia sativa* L.), soybean (*Soja max* (L.) Piper), crotalaria (*Crotalaria spectabilis* Roth), and sweet clover (*Melilotus alba* Desr.). Other plant species used in some of the investigations will be given in later papers.

Except for crotalaria and crown vetch, the plants just listed are common legumes, and it is therefore unnecessary to describe their nodules. The crotalaria nodules are of the vetch type and usually become branched after the first few days of growth. Crown vetch is not a true vetch although the leaves resemble hairy vetch to a marked extent. Its nodules resemble those of vetch except that they are usually less branched.

The legumes, with some exceptions, were grown in the field at the Experimental Farm at Arlington, Virginia. In most cases plantings were made at intervals in order that nodules of the desired type would be available when needed. Ordinarily only young active nodules from plants between the ages of two weeks and prior to flowering were used.

In many phases of these studies it would have been advantageous in successive experiments to use nodules from the same plant species that were of approximately the same age and in as nearly the same physiological condition as possible. This is obviously impossible when the nodules are grown under ordinary field conditions and experiments are conducted over a considerable period of time, since,

as our experience has repeatedly shown, the condition of nodules on a given plant may change markedly within a period of less than a week.

In the absence of standard or uniform nodular material, we chose to have growing at all times several species of legumes from which we could on any day select the nodules which seemed best suited to the experiment planned. One of the important results of the use of this procedure has been to show in a general way that, in so far as our observations extend, nodules of all legume species behave very much alike. For instance, if a given energy source is used by one type of nodule, as evidenced by an increase in the rate of respiration or an effect on respiratory quotient, nodules from other legume species will almost certainly also use the same substance. Nodules from different species may show quantitative variations but qualitatively they nearly always respond similarly. In fact, variations between different batches of nodules of the same species are ordinarily of the same order as those between different species, assuming that in both cases only nodules comparable in size and condition are used. Verification of these statements will be found in the tables included in this paper. It is desirable to emphasize these points in the beginning since frequently successive experiments have been made with different species of nodules.

**PREPARATION OF NODULES.**—The plants were dug carefully in the morning between the hours of 7:30 and 9:00, quickly transported to the laboratory, and washed. The nodules were picked off, again washed, sorted from defective ones (including all that floated in the wash water), dried between filter papers, weighed, and placed in the culture vessels. During most of the 1-4 hours that usually elapsed between the time of arrival of the plants at the laboratory and the beginning of the experiment, the nodules were kept in ice water or in the refrigerator at about 5° C.

The surfaces of the nodules were not sterilized before they were introduced into the respiration vessels, since in preliminary studies the few methods of sterilization tried reduced the rate of respiration appreciably.

When crushed nodules were used the method was to weigh out the usual quantity of nodules into the respiration vessels and crush

them with the blunt end of a glass rod. The nodules were not thoroughly ground up, therefore, but only broken and most of the contents squeezed out. The outer portions of the nodules, when of a tough structure, remained more or less intact as empty shells.

MANOMETRIC PROCEDURES.—When oxygen uptake and carbon dioxide production were measured, the usual procedure was to place 50–250 mg. (usually 150 mg.) of the fresh nodules in each Warburg vessel. Two cc. of basal medium containing the source of energy (if any), and usually without a nitrogen source, was then pipetted into the vessel. The basal medium described in detail elsewhere (1) was the same as used for the culture of rhizobia, and had the following composition:  $K_2HPO_4$ , 0.8 gm.;  $KH_2PO_4$ , 0.2 gm.; NaCl, 0.2 gm.;  $MgSO_4 \cdot 7H_2O$ , 0.2 gm.;  $CaSO_4 \cdot 2H_2O$ , 0.1 gm.;  $Fe_2(SO_4)_3 \cdot 9H_2O$ , 0.01 gm.; and  $H_2O$  (distilled) 1000 cc. The two phosphate salts were dissolved first and a solution of the other salts added. The medium was allowed to stand for several hours until the precipitate had settled out; then the clear solution was siphoned off as needed. Nitrogenous salts were not added except in a few cases mentioned. Glucose was supplied in about half the experiments, as shown in the tables. If oxygen consumption only was to be observed, 0.3 cc. of 2N KOH was placed in the center well of the vessel. In the few cases where samples of tissue greater than 200 mg. were used, half of the alkali was placed in the center well and the remainder in one side arm in order to increase the surface and thus aid rapid  $CO_2$  absorption. When, in addition to oxygen consumption, the carbon dioxide produced was to be recorded, the latter was absorbed in 0.4 cc. of saturated  $Ba(OH)_2$  solution placed in one of the side arms. In the other side arm 0.7 cc. of 2.5 N HCl was used. At the close of the experiment the hydrochloric acid was dumped first into the barium solution, decomposing the barium carbonate, and then into the nodules, stopping metabolic processes. During early work only 0.3 cc. of 2.5 N HCl was used, but it was found that with this amount of acid, evolution of carbon dioxide after acidification continued at a decreasing rate for considerable time. This difficulty in obtaining a sharp end point was overcome with only occasional exceptions by the use of the larger quantity of acid. Since the standard details of these

manometric procedures are given by DIXON (2), as well as by numerous other investigators, they need not be repeated here.

Repeated tests showed that experiments could ordinarily be carried out under non-sterile conditions for periods of 5-6 hours without bacterial action becoming significant. The respiration data reported in this and subsequent papers of the series are based upon periods of 2-6 hours, usually 3-5 hours. Oxygen consumption was recorded at intervals of 0.5-1 hour during the experiment, whereas carbon dioxide values were necessarily for the entire period.

ENERGY SOURCES.—During the early stages of these studies some eighteen energy sources, including mostly sugars, organic acids, and alcohols, were tested in a preliminary way for utilization by nodules. The ability of the plant tissues and bacteria to use these was judged by the effect on rate of oxygen consumption and respiratory quotient, but in many cases the results were neither clearly positive nor negative. Of the sugars used, glucose, as might be expected, seemed to give the best results and was used for practically all the work where a readily available source of energy was desired. A concentration of 1 or 2 per cent was usually employed.

It was found that nodules usually show about 80-95 per cent as high a rate of oxygen uptake in the absence of added glucose or other energy sources as in their presence. Hence "endogenous" respiration tends to mask the effect of added substrate and makes it difficult to determine manometrically whether it is utilized.

SIZE OF NODULES.—No very exact expression of nodule size is used in presenting the data. Nodules are designated merely as very small, small, medium, large, and very large. In using these terms cowpea nodules were considered as an approximate standard. If the diameters are expressed in millimeters the terms have the following approximate values: very small, 0.5-1.5; small, 1-2.5; medium, 1-4; large, 4-6; and very large, above 6. When small or medium sized nodules were used, the large ones were discarded but not the very small ones. This accounts for the wider spread in size in these two grades. Since some nodules, such as those of hairy vetch, are irregular in shape and branched, it is difficult to classify them; such large branched forms were discarded. The smaller young forms are

classified according to their approximate average diameters. The small nodules of the branched type often included some pieces of the larger ones.

The symbols used throughout this series of papers are defined as follows:

$Q_{O_2}$  = ml.  $O_2$  consumed per mg. dry weight of tissue per hour

$\bar{Q}_{O_2}$  = ml.  $O_2$  consumed per mg. moist weight of tissue per hour

$Q_{O_2}(N)$  = ml.  $O_2$  consumed per mg. tissue nitrogen per hour  
ml.  $CO_2$  produced

$R.Q. = \frac{\text{ml. } CO_2 \text{ produced}}{\text{ml. } O_2 \text{ consumed}}$

### Experimental results

During the course of these investigations about 700  $Q_{O_2}$  and 350 R.Q. determinations have been made to date, as well as a considerable but smaller number of other types of analyses. It is obviously impossible to report all these experiments in detail, hence the usual procedure will be to group together the data bearing on any point, no matter from what experiments secured. In many cases an experiment planned primarily for one given purpose may furnish excellent data on other points. This is one of the advantages of the standardized Warburg respiration technique. Ordinarily averages of two or more determinations, rather than individual values, will be given.

The rates of respiration of nodules are reported on the bases of moist weight, dry weight, and nitrogen content, except when the necessary data for calculation of some of these values were not obtained. In the discussion, reference will ordinarily be made to the  $Q_{O_2}$  values. The  $Q_{O_2}(N)$  values are of secondary interest in connection with the present study but are included for the sake of completeness. Special reference is made to these values in the second paper of this series.

#### RATES OF RESPIRATION AND RESPIRATORY QUOTIENTS OF WHOLE NODULES MAINTAINED IN VARIOUS PARTIAL PRESSURES OF OXYGEN IN NITROGEN

The data given in table 1 are taken from numerous experiments run at various times during the 4-year experimental period. A

TABLE 1

RATES OF RESPIRATION AND RESPIRATORY QUOTIENTS OF DETACHED NODULES  
FROM SEVERAL SPECIES OF LEGUMES MAINTAINED IN VARIOUS  
PARTIAL PRESSURES OF OXYGEN

EXPERI- MENT NO.	PLANT SPECIES	SIZE OF NODULES	GLU- COSE (%)	O <sub>2</sub> IN N <sub>2</sub> (%)	Q <sub>O<sub>2</sub></sub>	Q <sub>O<sub>2</sub></sub>	Q <sub>O<sub>2</sub></sub> (N)	R.Q.
11.....	Hairy vetch	Medium	0	21	0.563	5.63	.....	1.01
11.....	Hairy vetch	Medium	1	21	0.625	6.25	.....	1.00
51.....	Hairy vetch	Small	0	100	0.915	7.29	88.4	1.08
85.....	Hairy vetch	Very small	1	7.4	0.286	2.31	25.8	.....
85.....	Hairy vetch	Very small	1	21	0.526	4.24	47.4	.....
85.....	Hairy vetch	Very small	1	50	0.897	7.23	80.8	.....
85.....	Hairy vetch	Very small	1	100	1.108	8.93	99.8	.....
86.....	Hairy vetch	Small	0	100	1.220	8.16	108.5	1.11
86.....	Hairy vetch	Small	1	100	1.260	8.43	112.0	1.24
87.....	Hairy vetch	Small	0	21	0.570	3.83	57.7	1.15
87.....	Hairy vetch	Small	1	21	0.580	3.85	58.0	1.44
13.....	Crown vetch	Medium	0	21	0.281	.....	.....	0.97
13.....	Crown vetch	Medium	1	21	0.342	.....	.....	1.03
15.....	Crown vetch	Medium	0	21	0.357	2.03	33.1	1.23
15.....	Crown vetch	Medium	2	21	0.407	2.31	37.7	1.30
16.....	Crown vetch	Medium	0	21	0.380	2.41	35.0	1.15
16.....	Crown vetch	Medium	2	21	0.384	2.44	35.4	1.24
16.....	Crown vetch	Medium	0	100	0.797	5.06	73.5	1.08
17.....	Crown vetch	Medium	0	21	0.424	2.54	38.3	1.05
17.....	Crown vetch	Medium	2	21	0.447	2.68	38.9	1.09
18.....	Crown vetch	Medium	2	21	0.382	2.71	34.8	1.08
19.....	Crown vetch	Large	2	21	0.248	1.57	20.0	1.26
19.....	Crown vetch	Small	2	21	0.404	2.54	32.5	1.13
23.....	Crown vetch	Medium	2	21	0.304	1.91	24.4	1.17
23.....	Crown vetch	Medium	2	100	0.407	2.56	32.8	0.89
83.....	Crown vetch	Small	1	100	1.170	7.13	82.2	.....
84.....	Crown vetch	Small	1	100	1.210	8.29	100.9	.....
27.....	Common vetch	Small	0	21	0.347	2.80	38.5	1.00
27.....	Common vetch	Small	0	80	0.783	6.32	86.9	0.97
27.....	Common vetch	Small	2	7.4	0.210	1.69	23.3	1.30
27.....	Common vetch	Small	2	21	0.428	3.45	47.5	0.98
27.....	Common vetch	Small	2	50	0.711	5.73	79.0	1.01
27.....	Common vetch	Small	2	80	0.943	7.61	104.7	1.03
27.....	Common vetch	Small	2	100	0.970	7.82	107.7	1.02
65.....	Crotalaria	Small	0	100	0.749	5.74	72.0	.....
65.....	Crotalaria	Small	1	100	1.020	7.82	96.8	.....
66.....	Crotalaria	Small	0	21	0.353	2.30	29.2	.....
66.....	Crotalaria	Small	0	50	0.602	3.91	49.8	.....
66.....	Crotalaria	Small	0	100	0.813	5.28	67.2	.....
67.....	Crotalaria	Small	1	21	0.406	2.93	33.8	.....
67.....	Crotalaria	Small	1	50	0.711	5.13	59.3	.....
67.....	Crotalaria	Small	1	100	0.944	6.82	78.7	.....
70.....	Crotalaria	Small	1	100	0.971	7.25	86.6	.....
78.....	Crotalaria	Very small	0	100	0.873	6.19	69.7	0.95
78.....	Crotalaria	Very small	1	100	1.121	7.95	89.6	1.04
36.....	Sweet clover	Medium	0	100	0.652	3.83	85.7	1.03
36.....	Sweet clover	Medium	1	100	0.832	4.84	109.4	0.95
50.....	Sweet clover	Small	0	100	0.903	6.03	70.7	1.02
50.....	Sweet clover	Small	1	100	0.992	6.61	77.5	1.09
52.....	Sweet clover	Small	0	100	1.181	6.38	78.2	1.04

TABLE 1—Continued

EXPERIMENT NO.	PLANT SPECIES	SIZE OF NODULES	GLUCOSE (%)	O <sub>2</sub> IN N <sub>2</sub> (%)	Q <sub>O<sub>2</sub></sub>	Q <sub>O<sub>2</sub></sub>	Q <sub>O<sub>2</sub></sub> (N)	R.Q.
28.....	Lespedeza	Medium	0	21	0.281	1.69	22.8	1.17
28.....	Lespedeza	Medium	1	21	0.300	1.80	24.3	1.37
30.....	Lespedeza	Medium	0	21	0.269	1.58	19.8	1.11
30.....	Lespedeza	Medium	0	80	0.764	4.48	56.2	0.97
30.....	Lespedeza	Medium	0	100	0.855	5.02	62.8	0.94
30.....	Lespedeza	Medium	1	21	0.294	1.72	21.6	1.40
30.....	Lespedeza	Medium	1	80	0.875	5.13	64.3	1.03
30.....	Lespedeza	Medium	1	100	0.930	5.45	68.4	1.01
31.....	Lespedeza	Medium	0	21	0.257	1.50	19.1	1.26
31.....	Lespedeza	Medium	0	80	0.726	4.22	59.0	1.01
81.....	Lespedeza	Small	0	21	0.229	1.24	17.6	0.98
81.....	Lespedeza	Small	1	21	0.238	1.30	18.3	1.29
21.....	Soybean	Small	2	21	0.334	2.05	35.8	1.79
22.....	Soybean	Small	2	21	0.328	2.01	35.2	1.52
24.....	Soybean	Small	2	21	0.285	1.50	26.4	1.79
25.....	Soybean	Small	2	7.4	0.183	0.87	15.6	1.52
25.....	Soybean	Small	2	21	0.293	1.39	25.2	1.23
25.....	Soybean	Small	2	50	0.474	2.25	40.7	1.00
25.....	Soybean	Small	2	100	0.662	3.15	56.9	1.06
37.....	Soybean	Small	2	21	0.334	1.43	30.4	1.28
37.....	Soybean	Medium	2	21	0.301	1.28	27.3	.....
37.....	Soybean	Large	2	21	0.178	0.76	16.2	2.00
37.....	Soybean	Small	2	100	1.010	4.32	91.9	.....
37.....	Soybean	Medium	2	100	0.588	2.51	53.4	.....
37.....	Soybean	Large	2	100	0.288	1.23	26.2	1.16
38.....	Soybean	Small	2	100	0.967	4.19	88.0	1.03
39.....	Soybean	Large	2	100	0.501	1.98	45.1	.....
40.....	Soybean	Large	2	100	0.491	1.94	44.3	.....
41.....	Soybean	Large	2	21	0.153	0.62	13.4	.....
41.....	Soybean	Large	2	100	0.390	1.58	34.2	.....
42.....	Soybean	Large	2	21	0.248	1.01	22.7	.....
42.....	Soybean	Large	2	100	0.402	1.64	36.8	.....
1.....	Cowpea	Medium	0	50	0.394	.....	.....	.....
1.....	Cowpea	Medium	3	50	0.408	.....	.....	.....
6.....	Cowpea	Large	3	21	0.147	.....	.....	.....
49.....	Cowpea	Small	0	100	0.430	2.45	43.9	1.03
49.....	Cowpea	Small	1	100	0.488	3.78	49.8	1.04
55.....	Cowpea	Small	0	100	0.751	2.91	.....	1.03
55.....	Cowpea	Small	1	100	0.723	3.77	.....	1.00
56.....	Cowpea	Very small	0	100	0.600	3.24	.....	1.06
56.....	Cowpea	Very small	1	100	0.717	3.88	.....	1.11
57.....	Cowpea	Very small	0	100	0.703	3.74	.....	1.10
57.....	Cowpea	Very small	1	100	0.813	4.33	.....	1.06
68.....	Cowpea	Small	0	100	0.765	4.45	75.3	.....
68.....	Cowpea	Small	1	100	0.787	4.58	77.6	.....
71.....	Cowpea	Small	1	100	0.776	3.86	64.8	.....
75.....	Cowpea	Very small	0	100	1.050	5.80	100.2	1.06
75.....	Cowpea	Very small	1	100	1.153	6.37	110.0	1.10
79.....	Cowpea	Small	0	21	0.156	0.71	12.6	.....
79.....	Cowpea	Small	1	21	0.193	0.88	15.5	.....
80.....	Cowpea	Small	0	21	0.176	0.81	14.2	.....
80.....	Cowpea	Small	1	21	0.189	0.86	15.3	.....
82.....	Cowpea	Large	1	100	0.292	1.91	31.7	.....

typical set of time curves, drawn from data obtained in experiment no. 17 and showing the changes in rates of respiration during a 6-hour experimental period, is shown in figure 1. The effect of varying the partial pressure of oxygen upon the  $Q_{O_2}$  values of nodules is illustrated very strikingly in figure 2.

The rate curves in figure 1 illustrate some of the points already mentioned, particularly that glucose often has little effect upon the respiration of nodules. The curves also show that the rate of respira-

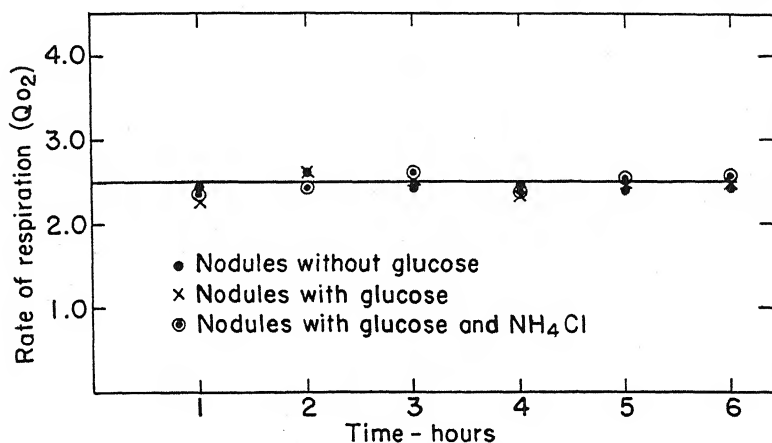


FIG. 1.—Rates of respiration of crown vetch nodules during 6-hour period maintained in various nutrient solutions in air.

tion of the nodules receiving ammonium chloride at a concentration of 100 ppm together with glucose was not significantly different from the rate with glucose alone. This observation was made in other experiments, hence combined nitrogen was omitted in all but a few of the respiration studies. It will be observed further that the respiration rate remains fairly constant over the 6-hour period of the experiment. If bacterial action had been an important factor, the respiration curve of course would have turned up sharply during the period of rapid bacterial growth. Occasionally the rate of respiration after a few hours will show a steady decrease instead of an increase. This decrease may sometimes be due to deficient energy supply in the nodules, but is more likely to occur when sugar is supplied, and when an increase in acidity occurs.

The  $Q_{O_2}$  values in air (table 1) varied from 0.62 to 6.25 for the

various plant species, with an average of about 2.2. The values in the presence of glucose, in the experiments where direct comparisons were made, were on the average about 12 per cent higher than in its absence. The young small nodules usually gave considerably higher

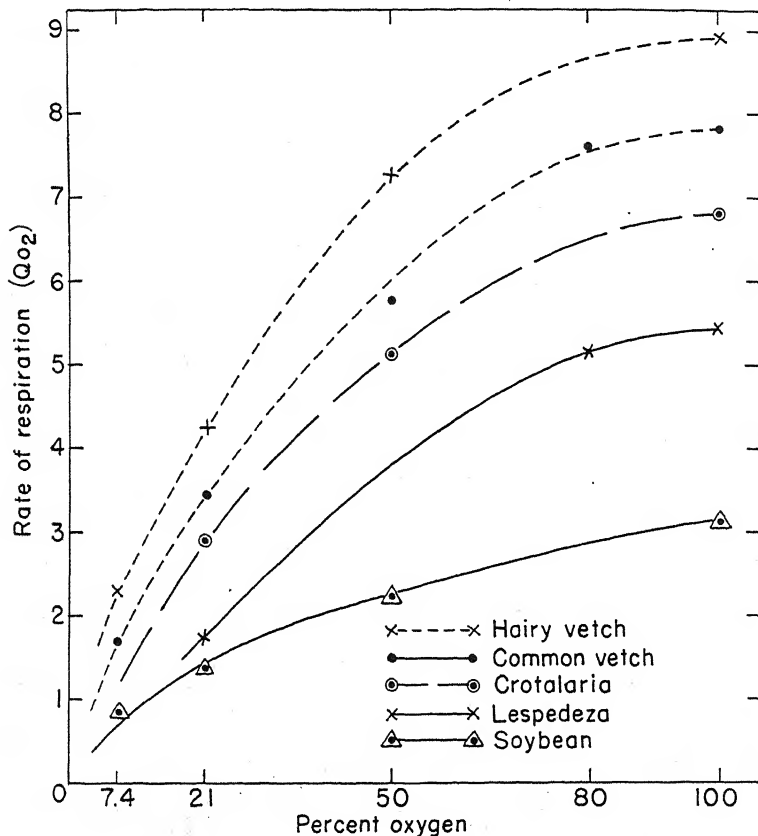


FIG. 2.—Rates of respiration of nodules from various species maintained in glucose medium in various partial pressures of oxygen.

values than the older and larger nodules, as would be expected. A relatively high or low value is usually not so much a characteristic of the plant species as of the size and condition of the nodule.

The  $Q_{O_2}$  values for nodules were markedly increased above the values in air by higher partial pressures of oxygen. An average of

the data from which figure 2 was drawn gives a  $QO_2$  of 2.75 in air and 6.43 in pure oxygen, an increase of 134 per cent even though only small or medium sized nodules were used. Likewise an average of all of the data in table 1, exclusive of the few experiments with large nodules, shows corresponding  $QO_2$  values for air and pure oxygen of 2.30 and 5.41, an increase of 135 per cent due to the additional oxygen. The addition of glucose to the nodules maintained in pure oxygen produced a mean increase in  $QO_2$  of 14 per cent over those given no carbohydrate.

The data of table 1 show that there is some tendency for the nodules of soybean, cowpea, and lespedeza to respire less rapidly than the nodules from the vetches, crotalaria, and sweet clover. This is probably due chiefly to the fact that the nodules from the first three species are usually less succulent and oxygen seems to penetrate less rapidly. If we compare the maximum, rather than the average,  $QO_2$  values observed for the nodules of the various plants as reported in table 1, the differences are somewhat less striking. The maximum  $QO_2$  values are for cowpea 6.4, lespedeza 5.5, and soybean 4.3; the values for the other plants are for hairy vetch 8.9, crown vetch 8.3, crotalaria 8.0, common vetch 7.8, and sweet clover 6.6. Apparently if sufficiently young succulent nodules are studied, the maximum  $QO_2$  values for all these species vary within a range of about twofold.

The respiratory quotients for nodules maintained in air and supplied with glucose show values ranging from approximately 1.0 to 2.0; in the absence of glucose the values were slightly lower (table 1). The theoretical R.Q. for the complete oxidation of glucose is of course 1.0. From these data, as well as from others not reported in table 1, it was noted that if the nodules were very small, young, and succulent, the respiratory quotients in the presence or absence of glucose were usually not far from the theoretical for glucose oxidation. When large nodules were used, however, the R.Q. values in air commonly reached a value of 1.7 or even higher. Such high values were most often obtained with cowpea and soybean nodules, owing in part to their larger size but also to the nature of the nodules. Apparently oxygen penetrates these nodules less rapidly than it does the vetch type of nodule.

The respiratory quotients, when much above 1.0 in air, usually show a marked decrease to near the theoretical value for glucose oxidation as the partial pressure of oxygen is increased. The R.Q. reaches a value of near 1.0 before the maximum  $QO_2$  is observed, indicating that even though the center of the nodule is not receiving the maximum amount of oxygen it is capable of using, the sugar is completely oxidized. In pure oxygen the R.Q. values, exclusive of those for the largest nodules, average about 1.03 and are not significantly different whether glucose was or was not added. In view of this and the fact that nodules are usually fairly well supplied with carbohydrates, it seems likely that glucose and probably sucrose, the more common sugars of most plant juices, are the chief energy sources used in normal nodule respiration. Of course other compounds often found in plant tissues have theoretical R.Q. values of near 1.0 and cannot be excluded as possible energy sources by the data presented here.

A number of factors influence the rate of oxygen consumption by plant and animal tissues. WARBURG (2, p. 50) early recognized the chief ones: (a) rate of respiration characteristic of the tissues being studied; (b) rate of diffusion of the oxygen through the tissues (which is a constant under a given oxygen pressure); (c) thickness of the portion of tissue used; and (d) concentration of oxygen immediately outside the tissue. It is obvious that in order to increase the oxygen supply inside a given nodule it is necessary to increase the partial pressure of  $O_2$  surrounding it or to resort to slicing or crushing in order to provide mechanically for oxygen entrance. The use of smaller nodules, as already pointed out, also permits better aeration, but such smaller nodules naturally represent material biologically different from the larger ones.

DIXON (2, p. 50) points out that it has been determined in studies with liver maintained in air that the slices must be less than 0.2 mm. thick in order for the center to receive adequate oxygen; in pure  $O_2$  the slices may be about 0.5 mm. in thickness. It is therefore not surprising that in table 1 most of the R.Q. values for nodules in air are above 1.0, even though few large ones were used. Even the smaller ones usually had a diameter of near 1.0 mm., and unless the diffusion constant of oxygen in nodular tissue is unusually high, or the

TABLE 2

RATES OF RESPIRATION OF CRUSHED NODULES FROM SEVERAL SPECIES  
OF LEGUMES MAINTAINED IN VARIOUS PARTIAL  
PRESSURES OF OXYGEN

EXPERI- MENT NO.	PLANT SPECIES	SIZE OF NODULES	TREAT- MENT	GLU- COSE (%)	OXY- GEN IN N (%)	Q <sub>O<sub>2</sub></sub>	Q <sub>O<sub>2</sub></sub>	Q <sub>O<sub>2</sub></sub> (N)
9.....	Hairy vetch	Medium	Whole	0	21	0.426	.....	.....
9.....	Hairy vetch	Medium	Whole	2	21	0.500	.....	.....
9.....	Hairy vetch	Medium	Crushed	0	21	0.525	.....	.....
9.....	Hairy vetch	Medium	Crushed	2	21	0.601	.....	.....
10.....	Hairy vetch	Medium	Whole	2	21	0.384	.....	.....
10.....	Hairy vetch	Medium	Crushed	2	21	0.501	.....	.....
85.....	Hairy vetch	Very small	Whole	1	7.4	0.286	2.31	25.8
85.....	Hairy vetch	Very small	Crushed	1	7.4	0.328	2.64	29.6
85.....	Hairy vetch	Very small	Whole	1	21	0.526	4.24	47.4
85.....	Hairy vetch	Very small	Crushed	1	21	0.384	3.10	34.6
85.....	Hairy vetch	Very small	Whole	1	50	0.897	7.23	80.8
85.....	Hairy vetch	Very small	Crushed	1	50	0.498	4.01	44.9
85.....	Hairy vetch	Very small	Whole	1	100	1.108	8.93	99.8
85.....	Hairy vetch	Very small	Crushed	1	100	0.472	3.80	42.5
83.....	Crown vetch	Small	Crushed	0	7.4	0.195	1.19	13.6
83.....	Crown vetch	Small	Crushed	1	7.4	0.222	1.35	15.6
83.....	Crown vetch	Small	Crushed	0	21	0.348	2.12	24.5
83.....	Crown vetch	Small	Crushed	1	21	0.338	2.06	23.8
83.....	Crown vetch	Small	Crushed	0	50	0.313	1.91	21.9
83.....	Crown vetch	Small	Crushed	1	50	0.483	2.94	33.8
83.....	Crown vetch	Small	Crushed	0	100	0.478	2.91	33.6
83.....	Crown vetch	Small	Crushed	1	100	0.522	3.18	36.6
83.....	Crown vetch	Small	Whole	1	100	1.170	7.13	82.2
84.....	Crown vetch	Small	Crushed	1	7.4	0.277	1.90	23.1
84.....	Crown vetch	Small	Crushed	1	21	0.360	2.47	30.0
84.....	Crown vetch	Small	Crushed	1	50	0.442	3.03	36.8
84.....	Crown vetch	Small	Crushed	1	100	0.484	3.31	40.3
84.....	Crown vetch	Small	Whole	1	100	1.210	8.29	100.9
65.....	Crotalaria	Small	Whole	0	100	0.749	5.74	72.0
65.....	Crotalaria	Small	Crushed	0	100	1.053	8.07	100.0
65.....	Crotalaria	Small	Whole	1	100	1.020	7.82	96.8
65.....	Crotalaria	Small	Crushed	1	100	1.140	8.74	108.2
70.....	Crotalaria	Small	Crushed	0	21	0.624	4.65	55.6
70.....	Crotalaria	Small	Crushed	1	21	0.733	5.47	65.4
70.....	Crotalaria	Small	Crushed	0	50	0.642	5.54	66.2
70.....	Crotalaria	Small	Crushed	1	50	0.815	6.08	72.7
70.....	Crotalaria	Small	Crushed	0	100	0.777	5.80	69.3
70.....	Crotalaria	Small	Crushed	1	100	0.921	6.87	82.1
70.....	Crotalaria	Small	Whole	1	100	0.971	7.25	86.6
9.....	Lespedeza	Medium	Whole	0	21	0.396	.....	.....
9.....	Lespedeza	Medium	Crushed	0	21	0.902	.....	.....
9.....	Lespedeza	Medium	Whole	2	21	0.391	.....	.....
9.....	Lespedeza	Medium	Crushed	2	21	0.924	.....	.....
1.....	Cowpea	Medium	Whole	3	50	0.408	.....	.....
1.....	Cowpea	Medium	Crushed	3	50	0.925	.....	.....
2.....	Cowpea	Medium	Whole	0	50	0.240	.....	.....
2.....	Cowpea	Medium	Crushed	0	50	0.524	.....	.....
2.....	Cowpea	Medium	Whole	1	50	0.224	.....	.....

TABLE 2—*Continued*

EXPERIMENT NO.	PLANT SPECIES	SIZE OF NODULES	TREATMENT	GLUCOSE (%)	OXYGEN IN N (%)	$\dot{Q}_{O_2}$	$Q_{O_2}$	$Q_{O_2}(N)$
2.....	Cowpea	Medium	Crushed	1	50	0.551	.....	.....
3.....	Cowpea	Medium	Crushed	1	50	0.760	.....	.....
58.....	Cowpea	Very small	Crushed	0	100	0.982	5.47	.....
58.....	Cowpea	Very small	Crushed	1	100	1.043	5.81	.....
71.....	Cowpea	Small	Crushed	1	21	0.733	3.64	61.1
71.....	Cowpea	Small	Crushed	1	50	0.964	4.80	80.3
71.....	Cowpea	Small	Crushed	1	100	1.116	5.55	93.2
71.....	Cowpea	Small	Whole	1	100	0.776	3.86	64.8
82.....	Cowpea	Small	Crushed	0	100	0.649	3.45	60.1
82.....	Cowpea	Small	Crushed	1	100	0.739	3.93	78.7
82.....	Cowpea	Large	Crushed	0	100	0.531	3.47	57.7
82.....	Cowpea	Large	Crushed	1	100	0.542	3.54	58.9
82.....	Cowpea	Large	Whole	1	100	0.292	1.91	31.7

$Q_{O_2}$  values very much lower than for liver, the nodules must be somewhat deficient in oxygen in the center.

When pure oxygen is used and the nodules are very small (about 1 mm. in diameter or less) they are apparently rather well aerated, and nearly maximum  $Q_{O_2}$  values usually will be observed. The center of larger nodules maintained in oxygen will, however, undoubtedly be deficient in this element. These findings are in fairly close agreement with expectations from the observations with liver, considering that liver usually has a  $Q_{O_2}$  two or three times that of nodules supplied adequate oxygen. Since in some of these experiments a portion of the nodules used exceeded 3 mm. in diameter, it could not be expected that maximum  $Q_{O_2}$  values would be reached, even in 100 per cent oxygen.

RATES OF RESPIRATION OF CRUSHED NODULES MAINTAINED  
IN VARIOUS PARTIAL PRESSURES OF OXYGEN  
IN NITROGEN

It is obvious that crushing produced somewhat variable results upon the rate of respiration (table 2). In general the  $Q_{O_2}$  was increased by crushing if the nodules were maintained in air, owing of course to the better aeration of the cells. Even in 100 per cent oxygen, crushing commonly produced a favorable effect upon the rate of respiration, if the nodules were of medium or larger size or were

of the type that did not allow oxygen to penetrate readily. In the majority of cases, however, if small nodules were crushed and maintained in pure oxygen, a marked decrease in  $QO_2$  occurred. Figure 3

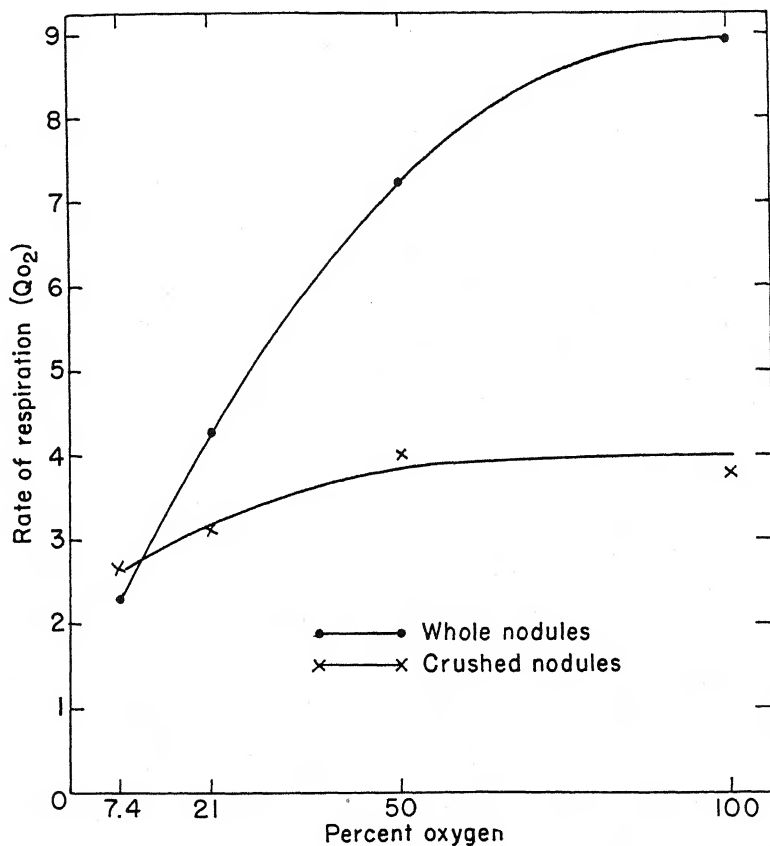


FIG. 3.—Rates of respiration of whole and crushed hairy vetch nodules maintained in various partial pressures of oxygen in nitrogen.

illustrates this point very well, although the harmful effect of crushing was unusually marked. A time curve showing the comparative  $QO_2$  values of crushed nodules in air and in pure oxygen, with and without added glucose, is given in figure 4. The decrease in  $QO_2$  during the first 3 hours was slightly more pronounced in pure oxygen. Evidently this method of increasing the aeration of the

inner cells of nodules is not very satisfactory if the observations continue for more than a few minutes.

The curves in figure 5 indicate that there is some stimulating effect of oxygen pressure, as such, on rate of respiration of crushed nodules. It would seem that such nodules would certainly be thoroughly aerated when constantly shaken in air. Nevertheless there was an increase of about 35 per cent in the  $Q_{O_2}$  values of such crushed

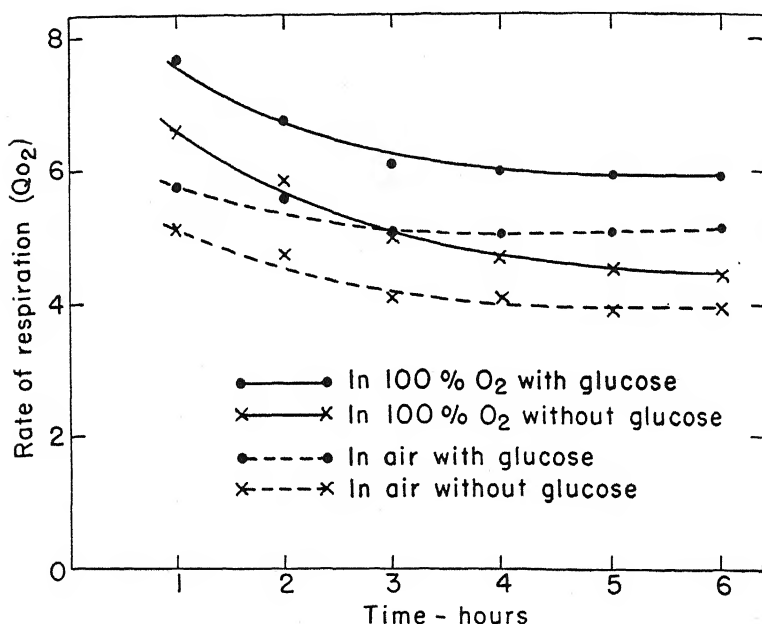


FIG. 4.—Rates of respiration of crushed crotonaria nodules in air and in 100 per cent oxygen in presence and absence of added glucose.

nodules when shaken in pure O<sub>2</sub> in comparison with the corresponding values for those kept in air. It should be remembered, however, that the nodules in these experiments were not completely macerated but merely broken and most of the contents squeezed out. Further experiments would be necessary to determine definitely whether the observed increase is a true oxygen concentration effect or merely an indication that even crushed nodules are still not adequately aerated for maximum oxygen consumption under the experimental conditions. The latter explanation seems somewhat more plausible.

# EFFECT OF GLUCOSE CONCENTRATION ON RESPIRATORY QUOTIENTS OF WHOLE NODULES

In some preliminary experiments conducted to determine the standard procedure to be followed in later work it was observed that sometimes glucose, particularly at concentrations of 5 per cent, slightly lowered the  $QO_2$  and increased the R.Q. values. In table 3

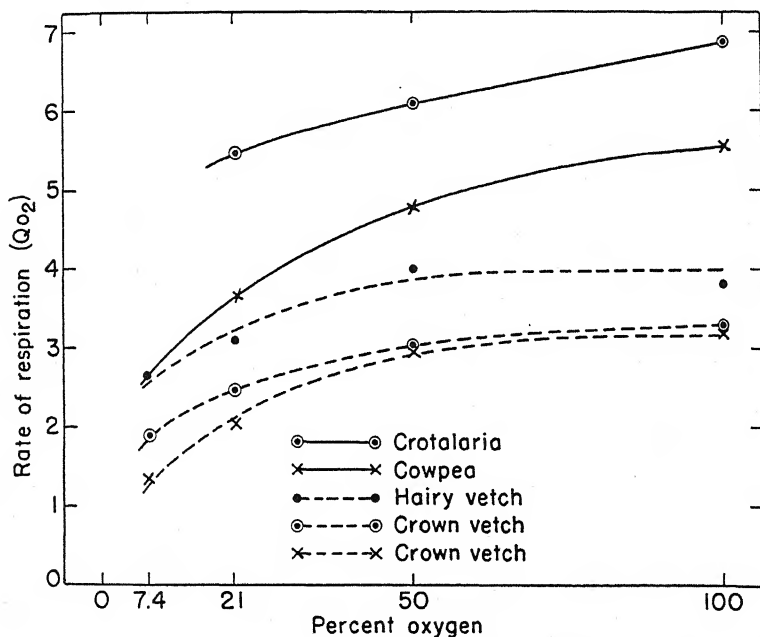


FIG. 5.—Effect of partial pressure of oxygen on rate of respiration of crushed nodules

are given data on the sugar effect at different partial pressures of oxygen. A portion of these results is plotted in figure 6.

In the experiments conducted in air, where the oxygen supply was inadequate for maximum rate of respiration, the R.Q. values were markedly increased by sugar, even at a concentration of 1 per cent, and still more so at higher percentages. The effect of the highest sugar concentration upon the  $QO_2$  values was usually not marked, although in a few cases they were lowered slightly. When 80 or 100 per cent  $O_2$  was substituted for air, however, the R.Q. values, even with 5 per cent glucose added, remained near 1.0 and the  $QO_2$  values were not significantly depressed.

These observations constitute further evidence for the view that the supply of oxygen inside the nodule maintained in air is limited. Under these conditions of deficient oxygen the addition of glucose in relatively high concentrations has the effect of increasing somewhat

TABLE 3

EFFECT OF GLUCOSE CONCENTRATION UPON THE RATE OF RESPIRATION  
AND RESPIRATORY QUOTIENTS AT DIFFERENT PARTIAL  
PRESSURES OF OXYGEN

EXPERI- MENT NO.	PLANT SPECIES	SIZE OF NODULES	GLU- COSE (%)	O <sub>2</sub> IN N <sub>2</sub> (%)	Q <sub>O<sub>2</sub></sub>	Q <sub>O<sub>2</sub></sub>	Q <sub>O<sub>2</sub></sub> (N)	R.Q.
28.....	Lespedeza	Medium	0	21	0.281	1.69	22.8	1.17
28.....	Lespedeza	Medium	1	21	0.300	1.80	24.3	1.37
28.....	Lespedeza	Medium	2.5	21	0.242	1.46	19.6	2.10
28.....	Lespedeza	Medium	5	21	0.202	1.21	16.3	2.43
29.....	Lespedeza	Medium	0	80	0.80	4.80	64.6	0.99
29.....	Lespedeza	Medium	1	80	0.85	5.11	68.8	1.03
29.....	Lespedeza	Medium	2.5	80	0.78	4.67	62.9	1.07
29.....	Lespedeza	Medium	5	80	0.84	5.06	68.2	1.11
30.....	Lespedeza	Medium	0	21	0.27	1.58	19.8	1.11
30.....	Lespedeza	Medium	1	21	0.29	1.72	21.6	1.40
30.....	Lespedeza	Medium	5	21	0.07	0.43	5.4	7.68*
30.....	Lespedeza	Medium	0	80	0.76	4.48	56.2	0.97
30.....	Lespedeza	Medium	1	80	0.88	5.13	64.3	1.03
30.....	Lespedeza	Medium	5	80	0.74	4.36	54.7	1.07
30.....	Lespedeza	Medium	0	100	0.86	5.02	62.8	0.94
30.....	Lespedeza	Medium	1	100	0.93	5.45	68.4	1.01
30.....	Lespedeza	Medium	5	100	0.86	5.04	63.2	1.07
81.....	Lespedeza	Small	0	21	0.229	1.24	17.6	0.98
81.....	Lespedeza	Small	1	21	0.238	1.30	18.3	1.29
81.....	Lespedeza	Small	2	21	0.262	1.43	20.2	1.38
81.....	Lespedeza	Small	3	21	0.247	1.35	19.0	1.49
81.....	Lespedeza	Small	4	21	0.196	1.07	15.1	1.53
81.....	Lespedeza	Small	5	21	0.248	1.35	19.1	1.63
87.....	Hairy vetch	Small	0	21	0.57	3.83	57.7	1.15
87.....	Hairy vetch	Small	1	21	0.58	3.85	58.0	1.44
87.....	Hairy vetch	Small	2	21	0.55	3.66	55.2	1.55
87.....	Hairy vetch	Small	3	21	0.57	3.79	57.1	1.54
87.....	Hairy vetch	Small	4	21	0.53	3.59	54.1	1.83
87.....	Hairy vetch	Small	5	21	0.53	3.53	53.1	1.66

\* This figure is the result of one determination only and is undoubtedly incorrect.

the evolution of CO<sub>2</sub>. The explanation for this probably involves a number of factors, but in the light of other data to be published later, it is highly probable that the abundant sugar supply favors a partially anaerobic breakdown of sugar inside the nodule. The outside layers of the nodule would presumably continue to use the available oxygen supply for normal aerobic oxidation of sugar, and

possibly of some of the intermediate products of anaerobic metabolism generated in the center of nodules. The net result of these two processes would of course be an increase in the R.Q., since the fermentative step would result in the formation of intermediate products and the evolution of  $\text{CO}_2$  with limited or no oxygen uptake.

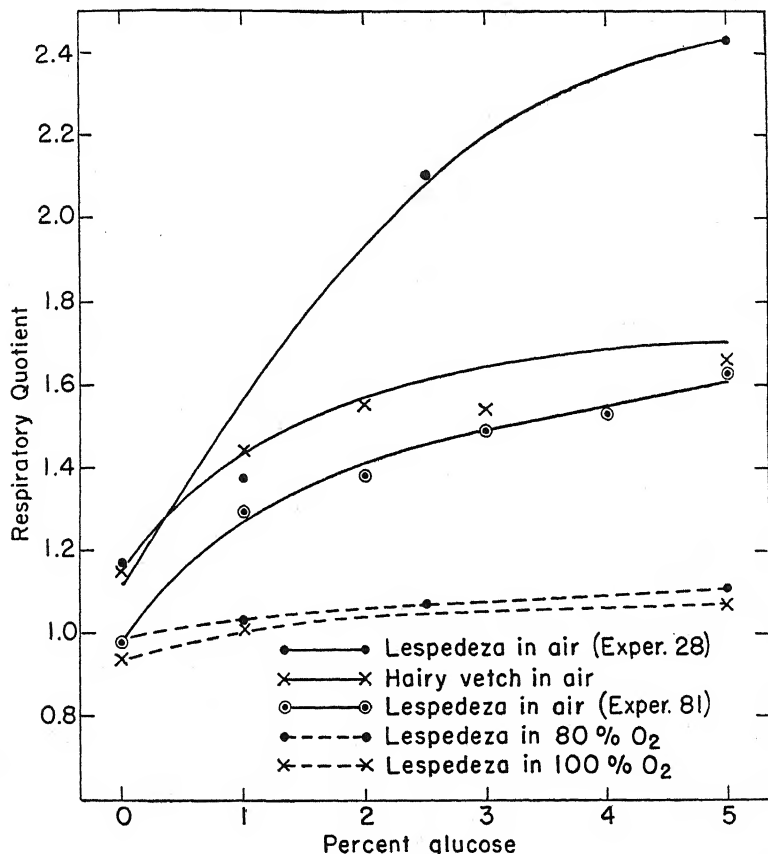


FIG. 6.—Effect of glucose concentration on respiratory quotient of nodules maintained in different partial pressures of oxygen.

### Discussion

Although these studies show that most detached nodules maintained in air in Warburg vessels have a limited internal oxygen supply, it should be borne in mind that such methods of experimentation necessarily represent artificial conditions. When attached

to the plant, the movement of dissolved oxygen into, and of  $\text{CO}_2$  from, the center of the nodule through the vascular strands may possibly be a significant factor. The direct diffusion of oxygen through the walls of the nodule under natural conditions may also be more rapid. In the experiments the nodules were constantly shaken in a thin layer of the basal medium (2 cc. per 100-200 mg. nodules), whereas in nature they are of course comparatively dry. In a few determinations on the rate of respiration of nodules kept in Warburg vessels without basal medium, the  $\text{QO}_2$  values were slightly lower than when the usual quantity of liquid medium was added. This indicates that oxygen penetration through the cortex of nodules under experimental conditions was as rapid as under field conditions. The penetration may well have been even more rapid, since in soils, particularly the heavier ones, air movement is retarded and in some cases the partial pressure of  $\text{O}_2$  in soil air may be appreciably less than in normal air.

While the facts indicate that under field conditions the oxygen supply within the center of most nodules, and particularly of the larger ones, is limited, it cannot be inferred that a strict anaerobic or fermentative metabolism necessarily occurs. As already pointed out, an R.Q. characteristic of aerobic glucose utilization is frequently obtained under conditions where the oxygen supply is inadequate for maximum  $\text{QO}_2$  values. This indicates that true fermentation does not occur to any appreciable extent until the oxygen supply is rather limiting, or else if a fermentative step does occur the intermediate products are later oxidized to  $\text{CO}_2$  and water.

Since nitrogen fixation occurs in nodules in air, where the  $\text{O}_2$  concentration is not over 21 per cent and may be somewhat less, and commonly in nodules clearly larger than the largest which would give an R.Q. in air as low as unity, the conclusion seems evident that it occurs normally under partial anaerobiosis. These considerations constitute in no sense a denial of the often observed importance of soil aeration to legumes, but merely call attention to the condition within the nodule, where fixation occurs.

### Summary

Respiration studies were undertaken with a view to obtaining fundamental information concerning the metabolic processes of

legume nodules, inasmuch as the knowledge gained should facilitate investigation of the process of nitrogen fixation. The results obtained by standard manometric procedures with detached nodules from eight species of legumes may be summarized as follows:

1. The rates of respiration of nodules maintained in various oxygen-nitrogen mixtures increased as the oxygen percentage increased. The  $QO_2$  values varied between 0.62 and 6.25 with an average of about 2.2 in air, and were about 2.4 times as great in pure oxygen, with maxima for the eight species varying between 4.3 for soybean and 8.9 for hairy vetch.

2. The respiratory quotients increased with increase in size of nodule and with decrease in concentration of oxygen. In air the values varied between 1.0 and 2.0, depending largely on the size of the nodules. In pure oxygen, excluding very large nodules, the values averaged 1.03.

3. The addition of glucose to the nutrient medium produced a mean increase in  $QO_2$  of about 12 per cent in air and 14 per cent in 100 per cent oxygen. It increased the R.Q. at the lower concentrations of oxygen but not appreciably in pure oxygen.

4. These results with detached nodules are interpreted as indicating that with most nodules maintained in air, or with large nodules at oxygen concentrations even as high as 100 per cent, the interior of the nodules is under anaerobic or partially anaerobic conditions. The evidence further indicates that under natural conditions in the soil the oxygen supply inside most nodules is also limited.

5. Crushed nodules gave higher  $QO_2$  values when maintained in air than did whole nodules, but usually lower values than whole nodules in pure oxygen. This method of increasing the aeration of the inner cells of the nodules was not very satisfactory.

6. The nodules of all the species showed rather similar responses, the variations between species being not much greater than that within species.

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# BIOCHEMICAL NITROGEN FIXATION STUDIES

## II. COMPARATIVE RESPIRATION OF NODULES AND ROOTS, INCLUDING NON-LEGUME ROOTS

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(WITH TWO FIGURES)

### Introduction

In the preceding paper (3) of this series data were presented showing the rates of respiration and respiratory quotients of various species and sizes of detached legume nodules maintained in various partial pressures of oxygen in nitrogen. These studies showed  $Q_{O_2}$  values averaging about 2.2 in air and 5.4 in pure oxygen. The R.Q. values were near 1.0 for nodules maintained in pure oxygen but frequently considerably higher than this when kept in air. It was concluded that the supply of oxygen inside most nodules maintained in air is limited.

The purpose of the present study was to determine to what extent nodule metabolism differs from root metabolism, particularly that of legume roots. Since nodules are filled with bacteria there seems to be a rather widespread belief that their metabolism, as evidenced by rate of respiration, is markedly higher than that of root tissues.

### Methods

The methods used were essentially as described in the preceding paper and consisted for the most part of the standard manometric procedures (4).

In addition to the legumes listed previously (3), a limited amount of work was done with white clover (*Trifolium repens* L.) and lima bean (*Phaseolus lunatus* L.). The non-legume species studied included corn (*Zea mays* L.), oat (*Avena sativa* L.), potato (*Solanum*

<sup>1</sup> The writers are indebted to ELLEN K. RIST of this laboratory for the nitrogen analyses and to E. C. BUTTERFIELD of the Bureau of Plant Industry for supplying much of the plant material.

*tuberosum* L.), tomato (*Lycopersicon esculentum* Mill.), cabbage (*Brassica oleracea* L.), crabgrass (*Digitaria sanguinalis* (L.) Scop.), pigweed (*Amaranthus retroflexus* L.), narrow leaved plantain (*Plantago lanceolata* L.), broad leaved plantain (*P. rugelii* Dcne.), ragweed (*Ambrosia artemisiaefolia* L.), cocklebur (*Xanthium* sp.), and jimson weed (*Datura* sp.).

The nodules were obtained from plants in an active vegetative stage and were prepared for use as described in the preceding paper. When comparative studies of the respiration of nodules and legume roots were made, the root samples were, with few exceptions, taken from the same batch of plants as were the nodules. The non-legume roots were obtained from young plants of approximately similar age and condition, and usually from plants grown on the same type of soil as the legumes. The same procedure was followed in preparing them for use as in the case of the nodules. They were washed thoroughly, most of the adherent water removed by placing between filter papers, and cut into lengths of 0.5–1 cm. to permit more uniform sampling and easy introduction into the small respiration vessels. After bringing the plants into the laboratory they were kept in ice water or in a refrigerator at about 5° C. for as much as possible of the time that necessarily elapsed before starting the experiment. The studies with both roots and nodules were carried out with unsterilized material. Most of the  $QO_2$  and R.Q. results are based upon periods of 2–6 hours (usually 3–5 hours), during which time bacterial action was not appreciable. The usual size of the nodules and root samples was 150–200 mg. moist weight in 2 cc. of the usual basal medium (3), containing minerals but no nitrogen, and with or without glucose as desired.

In the frequent references to the comparative rates of respiration of small nodules and small roots which follow it is not meant to imply that the two types of plant material are of like diameter. Actually the small nodules had a diameter of about 0.5–2.5 mm. whereas the small roots seldom exceeded 1 mm. in diameter and a considerable portion was near 0.1 mm. This means, of course, that when maintained in air the small roots were much better supplied with oxygen than were the small nodules.

### Experimental results

In reporting and interpreting the results, the plan used previously (3) of grouping all the pertinent data from a large number of experiments that deal with a given variable will be followed. In many of the earlier experiments, dealing primarily with nodule respiration, it was common to use roots in one or two of the respiration vessels as a control upon the nodule results, as well as to obtain a general idea of how nearly they behave like nodules. In later work many experiments dealt exclusively with roots. Rates of respiration, as in the preceding paper, are reported in terms of ml.  $O_2$  consumed per mg. dry weight,  $QO_2$ ; per mg. moist weight,  $\dot{Q}O_2$ ; and per mg. of tissue nitrogen,  $QO_2(N)$ . The figures as given are with few exceptions the averages of two or more determinations.

#### RATES OF RESPIRATION AND RESPIRATORY QUOTIENTS OF NODULES AND LEGUME ROOTS MAINTAINED IN VARIOUS PARTIAL PRESSURES OF OXYGEN IN NITROGEN

In table 1 are shown the  $QO_2$  values for the roots of several species of legumes maintained in nutrient solutions in various partial pressures of oxygen in nitrogen. A few values for nodules, when direct comparisons were made, are also included. A portion of these data are plotted in figure 1. Fairly typical time curves (experiment no. 68) showing the course of respiration of roots and nodules with and without glucose are shown in figure 2. In correlating the root respiration results with the corresponding values for nodules, it is necessary to bear in mind not only the data on nodules reported in table 1 but also the results presented in the first paper of this series.

The  $QO_2$  values for roots in air (table 1) varied between 0.57 (for a sample of large roots) and 4.19, with an average of 2.1. There was no marked difference between the  $QO_2$  values for roots and for the few samples of nodules reported here. In the previous paper the average  $QO_2$  value reported for a large number of samples of nodules maintained in air was 2.2. The rate of oxygen consumption per milligram of undried tissue ( $\dot{Q}O_2$ ) was somewhat higher with nodules than with roots, however, because young succulent roots ordinarily have a higher moisture content. Discussion of the  $QO_2(N)$  data will be given in a later section of this paper.

TABLE 1

COMPARATIVE RATES OF RESPIRATION AND RESPIRATORY QUOTIENTS OF  
LEGUME ROOTS AND NODULES MAINTAINED IN VARIOUS PARTIAL  
PRESSURES OF OXYGEN IN NITROGEN

EXPERI- MENT NO.	PLANT SPECIES	PLANT PART	SIZE	GLU- COSE (%)	O <sub>2</sub> IN N <sub>2</sub> (%)	Q <sub>O<sub>2</sub></sub>	Q <sub>O<sub>2</sub></sub>	Q <sub>O<sub>2</sub></sub> (N)	R.Q.
8.....	Crown vetch	Nodules	Medium	3	21	0.213	.....	.....	.....
8.....	Crown vetch	Roots	Medium	3	21	0.207	.....	.....	.....
14.....	Crown vetch	Nodules	Medium	0	21	0.253	.....	.....	0.97
14.....	Crown vetch	Roots	Medium	0	21	0.271	.....	.....	1.08
14.....	Crown vetch	Roots	Medium	1	21	0.330	.....	.....	1.10
73.....	Crown vetch	Roots	Small	0	50	0.269	1.63	69.2	.....
73.....	Crown vetch	Roots	Small	1	50	0.299	1.80	76.6	.....
65.....	Crotalaria	Nodules	Small	0	100	0.749	5.74	72.0	.....
65.....	Crotalaria	Nodules	Small	1	100	1.020	7.82	96.8	.....
65.....	Crotalaria	Roots	Small	0	100	0.276	2.60	125.0	.....
65.....	Crotalaria	Roots	Small	1	100	0.376	3.55	170.5	.....
65.....	Crotalaria	Roots	Large	0	100	0.196	1.29	100.8	.....
65.....	Crotalaria	Roots	Large	1	100	0.317	2.09	163.3	.....
66.....	Crotalaria	Nodules	Small	0	21	0.353	2.30	29.2	.....
66.....	Crotalaria	Roots	Small	0	21	0.223	2.10	93.1	.....
66.....	Crotalaria	Nodules	Small	0	50	0.602	3.91	49.8	.....
66.....	Crotalaria	Roots	Small	0	50	0.219	2.06	91.4	.....
66.....	Crotalaria	Nodules	Small	0	100	0.813	5.28	67.2	.....
66.....	Crotalaria	Roots	Small	0	100	0.215	2.02	89.6	.....
66.....	Crotalaria	Roots	Small	1	100	0.300	2.82	125.4	.....
67.....	Crotalaria	Nodules	Small	1	21	0.406	2.93	33.8	.....
67.....	Crotalaria	Roots	Small	1	21	0.244	2.44	125.2	.....
67.....	Crotalaria	Nodules	Small	1	50	0.711	5.13	59.3	.....
67.....	Crotalaria	Roots	Small	1	50	0.269	2.69	137.9	.....
67.....	Crotalaria	Nodules	Small	1	100	0.944	6.82	78.7	.....
67.....	Crotalaria	Roots	Small	1	100	0.269	2.69	137.9	.....
67.....	Crotalaria	Roots	Small	0	100	0.195	1.95	100.0	.....
68.....	Crotalaria	Roots	Small	1	100	0.252	2.54	116.5	.....
69.....	Sweet clover	Roots	Small	0	21	0.305	1.99	67.5	.....
69.....	Sweet clover	Roots	Small	1	21	0.405	2.65	90.0	.....
76.....	White clover	Roots	Small	0	21	0.251	1.84	88.9	.....
76.....	White clover	Roots	Small	1	21	0.296	2.17	104.8	.....
76.....	White clover	Roots	Small	1	100	0.309	2.27	109.7	.....
77.....	Lima bean	Roots	Small	0	21	0.225	2.61	94.1	.....
77.....	Lima bean	Roots	Small	1	21	0.306	3.53	127.5	.....
77.....	Lima bean	Roots	Small	1	100	0.509	5.88	212.4	.....
32.....	Lespedeza	Roots	Small	0	21	0.271	1.50	75.1	0.80
32.....	Lespedeza	Roots	Small	1	21	0.424	2.35	117.5	0.97
32.....	Lespedeza	Roots	Small	0	80	0.372	2.06	103.0	0.68
32.....	Lespedeza	Roots	Small	1	80	0.543	3.01	150.4	0.94
32.....	Lespedeza	Nodules	Medium	1	80	0.782	4.74	57.1	1.06
33.....	Lespedeza	Roots	Medium	0	21	0.300	1.64	88.1	0.85
33.....	Lespedeza	Roots	Medium	0	80	0.293	1.60	86.1	0.79
34.....	Lespedeza	Roots	Medium	0	21	0.331	1.51	80.8	.....
35.....	Lespedeza	Roots	Medium	0	21	0.282	1.49	81.4	0.94
35.....	Lespedeza	Roots	Medium	1	21	0.379	2.01	109.8	0.94
69.....	Lespedeza	Roots	Small	0	21	0.202	1.14	65.5	.....
69.....	Lespedeza	Roots	Small	1	21	0.340	1.92	110.3	.....

TABLE 1—*Continued*

EXPERIMENT NO.	PLANT SPECIES	PLANT PART	SIZE	GLUCOSE (%)	O <sub>2</sub> IN N <sub>2</sub> (%)	Q <sub>O<sub>2</sub></sub>	Q <sub>O<sub>2</sub></sub>	Q <sub>O<sub>2</sub></sub> (N)	R.Q.
21.....	Soybean	Nodules	Small	2	21	0.334	2.05	35.8	1.79
21.....	Soybean	Roots	Small	2	21	0.162	2.76	48.3	.....
22.....	Soybean	Nodules	Small	2	21	0.146	0.89	15.6	.....
22.....	Soybean	Roots	Small	2	21	0.131	2.50	48.4	.....
38.....	Soybean	Roots	Large	2	21	0.138	0.57	50.4	1.33
38.....	Soybean	Roots	Small	2	21	0.343	1.92	114.3	0.87
38.....	Soybean	Roots	Small	2	100	0.369	2.06	122.6	1.00
38.....	Soybean	Roots	Large	2	100	0.250	1.04	91.4	.....
38.....	Soybean	Nodules	Small	2	100	0.967	4.19	88.0	1.03
39.....	Soybean	Nodules	Large	2	100	0.501	1.98	45.1	.....
39.....	Soybean	Roots	Large	2	100	0.382	1.69	110.7	.....
1.....	Cowpea	Nodules	Medium	0	50	0.394	.....	.....	.....
1.....	Cowpea	Roots	.....	0	50	0.312	.....	.....	.....
1.....	Cowpea	Nodules	Medium	3	50	0.408	.....	.....	.....
1.....	Cowpea	Roots	.....	3	50	0.341	.....	.....	.....
2.....	Cowpea	Nodules	Medium	1	50	0.224	.....	.....	.....
2.....	Cowpea	Roots	.....	1	50	0.117	.....	.....	.....
3.....	Cowpea	Nodules	Medium	1	50	0.760	.....	.....	.....
3.....	Cowpea	Roots	.....	1	50	0.226	.....	.....	.....
6.....	Cowpea	Nodules	Medium	3	21	0.094	.....	.....	.....
6.....	Cowpea	Roots	.....	3	21	0.097	.....	.....	.....
7.....	Cowpea	Nodules	Small	3	21	0.205	.....	.....	.....
7.....	Cowpea	Roots	.....	3	21	0.192	.....	.....	.....
68.....	Cowpea	Nodules	Small	0	100	0.765	4.45	75.3	.....
68.....	Cowpea	Nodules	Small	1	100	0.787	4.58	77.6	.....
68.....	Cowpea	Roots	Small	0	100	0.362	3.35	122.7	.....
68.....	Cowpea	Roots	Small	1	100	0.529	4.90	179.3	.....
71.....	Cowpea	Nodules*	Small	1	21	0.733	3.64	61.1	.....
71.....	Cowpea	Roots	Small	1	21	0.455	4.19	197.8	.....
71.....	Cowpea	Nodules*	Small	1	50	0.964	4.80	80.3	.....
71.....	Cowpea	Roots	Small	1	50	0.587	5.34	255.3	.....
71.....	Cowpea	Nodules*	Small	1	100	1.116	5.55	93.2	.....
71.....	Cowpea	Roots	Small	1	100	0.683	6.21	297.1	.....
71.....	Cowpea	Nodules	Small	1	100	0.776	3.86	64.8	.....

\* Nodules in these vessels were crushed.

An increase in oxygen pressure above that in air had, on the average, a much smaller effect upon the Q<sub>O<sub>2</sub></sub> values of roots than of nodules. The average Q<sub>O<sub>2</sub></sub> value for roots kept in pure oxygen was 2.88, an increase of only about 35 per cent over the mean value in air, whereas the corresponding increase in nodular respiration (3) was about 135 per cent. The explanation for the difference depends upon the fact that nodules ordinarily are capable of a higher maximum respiration rate than are roots, but under ordinary atmospheric conditions not enough oxygen for these higher rates can diffuse into

the nodules. If roots are used that have diameters as great as those of nodules and have very low  $QO_2$  values in air, then there is also a marked increase in rate of respiration with increase in oxygen pressure. The maximum  $QO_2$  values observed for such roots are of course seldom as high as for nodules. Sometimes even small roots which happen to be relatively impervious to oxygen also show a marked response to increased oxygen pressure. The results obtained with

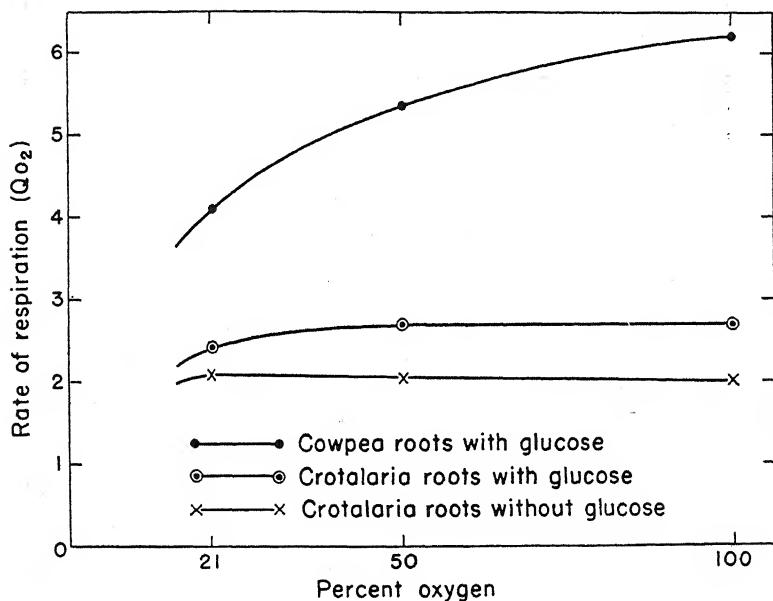


FIG. 1.—Effect of partial pressure of oxygen on rates of respiration of legume roots

small cowpea roots are an excellent example (fig. 1). As shown in the same figure, however, small crotalaria roots showed essentially no response to the additional oxygen.

A comparison of the rates of respiration of the roots of the various species of legumes shows no very marked differences for roots of comparable size, age, and condition. This is in agreement with the previous findings with regard to the  $QO_2$  values of nodules from various plant species.

The respiratory quotients of small roots maintained in nutrient solutions in air averaged 0.92 in the absence of added glucose and

0.97 when it was present. The values were therefore definitely lower than for nodules, owing primarily to the larger diameter and greater oxygen lack in the latter. Large roots also showed R.Q. values considerably higher than 1.0 but not so high as for the larger nodules.

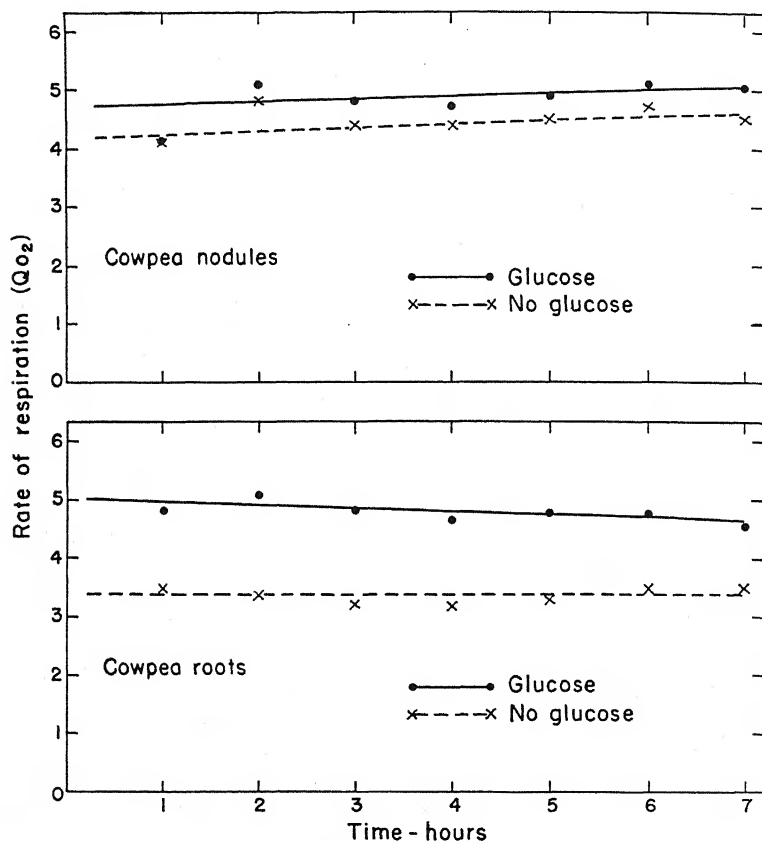


FIG. 2.—Rates of respiration of cowpea nodules and roots in 100 per cent oxygen in presence and absence of glucose.

Only a few R.Q. values were obtained with legume roots maintained in partial pressures of oxygen greater than in air, but these data show values somewhat lower than in air.

The data in table 1 indicate that legume roots are ordinarily more deficient in immediately available carbohydrates than are nodules.

As shown in table 1, the addition of glucose to roots increased the rate of respiration an average of about 40 per cent when direct comparisons were made. The beneficial effect of glucose was slightly greater in pure oxygen than in air. The increase with nodules due to glucose, as pointed out previously (3), was on the average about 12-14 per cent and was sometimes almost negligible. These observations are in harmony with the well known fact that young roots are essentially conducting vessels and relatively free from storage materials, whereas nodules are high in protein and not infrequently contain considerable starch.

A general summary of the results leads to the definite conclusion that young active nodules maintained in air usually respire at near the same rate per unit of dry matter as young succulent roots. But nodules have a capacity for respiring two or three times faster than roots if they are placed in pure oxygen so as to provide for more rapid penetration of the oxygen into the centers that are normally partially anaerobic. Of course this does not happen except under laboratory conditions, hence the  $QO_2$  values of young roots and nodules in nature are probably not greatly different.

#### RATES OF RESPIRATION OF LEGUME AND NON-LEGUME ROOTS

The determination that legume nodules and root tissues behave much alike with regard to rates of respiration per unit dry weight under normal atmospheric conditions led to the inquiry as to whether there is any marked difference between legume and non-legume root tissues. There is need for quantitative data on this point, since there seems to be a rather general impression (1, p. 129), based upon limited quantitative data obtained in part from measurements made in the field, that legume roots respire considerably more rapidly than do non-legume roots. This view is not, however, universal. For instance, SMITH (6) found that legumes produce no more  $CO_2$  than do other crops. Table 2 gives data on this point for seven species of legumes and twelve of non-legumes.

The  $QO_2$  values for small legume roots maintained in air, including the values given in table 1, varied between 1.14 and 4.19 with an average of about 2.2; the corresponding values for the non-legumes varied between 1.03 and 3.67 with an average of 2.3. There was

TABLE 2

COMPARISON OF RATES OF RESPIRATION OF SMALL  
LEGUME AND NON-LEGUME ROOTS

EXPERIMENT NO.	PLANT SPECIES	GLU- COSE (%)	O <sub>2</sub> IN N <sub>2</sub> (%)	Q <sub>O<sub>2</sub></sub>	Q <sub>O<sub>2</sub></sub>	Q <sub>O<sub>2</sub></sub> (N)	R.Q.
68.....	Cowpea	0	100	0.362	3.35	122.7	.....
68.....	Cowpea	1	100	0.529	4.90	179.3	.....
68.....	Corn	0	100	0.244	2.89	173.7	.....
68.....	Corn	1	100	0.319	3.78	227.4	.....
68.....	Crotalaria	1	100	0.252	2.54	116.5	.....
69.....	Sweet clover	0	21	0.305	1.99	67.5	.....
69.....	Sweet clover	1	21	0.405	2.65	90.0	.....
69.....	Lespedeza	0	21	0.202	1.14	65.5	.....
69.....	Lespedeza	1	21	0.340	1.92	110.3	.....
69.....	Pigweed	0	21	0.248	2.48	95.4	.....
69.....	Pigweed	1	21	0.367	3.67	141.2	.....
69.....	Tomato	0	21	0.204	1.92	101.6	.....
69.....	Tomato	1	21	0.276	2.61	138.1	.....
69.....	Oat	0	21	0.121	1.21	73.3	.....
69.....	Oat	1	21	0.166	1.66	100.6	.....
69.....	Crab grass	0	21	0.211	2.07	101.5	.....
69.....	Crab grass	1	21	0.336	3.29	161.3	.....
69.....	Narrow leaved plantain	1	21	0.142	1.03	143.0	.....
73.....	Crown vetch	0	50	0.269	1.63	69.2	.....
73.....	Crown vetch	1	50	0.299	1.80	76.6	.....
73.....	Ragweed	0	50	0.168	2.16	106.2	.....
73.....	Ragweed	1	50	0.211	2.70	133.0	.....
73.....	Broad leaved plantain	0	50	0.119	1.31	92.9	.....
73.....	Broad leaved plantain	1	50	0.196	2.16	153.2	.....
76.....	White clover	0	21	0.251	1.84	88.9	.....
76.....	White clover	1	21	0.266	2.17	104.8	.....
76.....	Cabbage	0	21	0.289	2.00	83.4	.....
76.....	Cabbage	1	21	0.370	2.55	106.3	.....
76.....	Potato	0	21	0.207	2.35	92.2	.....
76.....	Potato	1	21	0.299	3.40	133.3	.....
76.....	White clover	1	100	0.309	2.27	109.7	.....
76.....	Cabbage	1	100	0.387	2.67	111.3	.....
76.....	Potato	0	100	0.183	2.07	81.2	.....
76.....	Potato	1	100	0.303	3.44	134.9	.....
77.....	Lima bean	0	21	0.225	2.61	94.1	.....
77.....	Lima bean	1	21	0.306	3.53	127.5	.....
77.....	Cocklebur	0	21	0.137	1.98	85.9	.....
77.....	Cocklebur	1	21	0.232	3.34	145.9	.....
77.....	Jimson weed	0	21	0.130	1.84	69.6	.....
77.....	Jimson weed	1	21	0.252	3.55	134.5	.....
77.....	Lima bean	1	100	0.509	5.88	212.4	.....
77.....	Cocklebur	1	100	0.217	3.12	135.5	.....
77.....	Jimson weed	0	100	0.114	1.61	61.0	.....
77.....	Jimson weed	1	100	0.258	3.63	137.4	.....
53.....	Oat	0	100	0.206	1.75	125.0	0.92
53.....	Oat	1	100	0.242	2.05	146.4	0.96
54.....	Oat	0	100	0.261	1.94	137.3	0.92
54.....	Oat	1	100	0.295	2.19	155.1	0.90

TABLE 2—*Continued*

EXPERIMENT NO.	PLANT SPECIES	GLUCOSE (%)	O <sub>2</sub> IN N <sub>2</sub> (%)	Q <sub>O<sub>2</sub></sub>	Q <sub>O<sub>2</sub></sub>	Q <sub>O<sub>2</sub></sub> (N)	R.Q.
72.....	Corn	0	21	0.123	1.35	75.0	0.97
72.....	Corn	1	21	0.162	1.77	98.3	1.04
72.....	Corn	0	100	0.121	1.32	73.7	0.88
72.....	Corn	1	100	0.158	1.73	95.8	0.91
72.....	Corn*	1	100	0.183	2.32	133.3	1.00
74.....	Corn	0	21	0.178	1.78	153.5	0.89
74.....	Corn	1	21	0.206	2.06	177.6	0.95
74.....	Corn	0	100	0.181	1.81	155.6	0.92
74.....	Corn	1	100	0.229	2.29	197.0	0.91

\* Very young succulent roots.

obviously no significant difference between the rates of respiration of the small legume and small non-legume roots, although, as would be expected, there were some species differences in the case of both groups of plants. Not enough determinations were made, however, to make certain that the values obtained were truly characteristic of species and not merely differences in the particular root samples studied. On the whole the  $Q_{O_2}$  values for the twelve plant species varied within a comparatively narrow range.

When pure oxygen was used, the  $Q_{O_2}$  values for the non-legume roots (table 2) averaged 2.4, whereas the corresponding value for the legume roots (table 1) was 2.9. It is probable that the small increase in  $Q_{O_2}$  usually produced by increased oxygen pressure in both legume and non-legume roots is due only to the better aeration of inside cells.

The respiratory quotients of corn and oat roots (table 2) show average values for results obtained in air and in 100 per cent oxygen of 0.92 for roots not receiving glucose and 0.95 for those supplied with 1 per cent glucose. These non-legume roots therefore responded much like legume roots. In both cases the R.Q. values were lower than for nodules. The frequent failure to obtain R.Q. values of 1.0 with both legume and non-legume roots suggests that in the absence of added sugars these roots may have been oxidizing appreciable quantities of materials other than sugars, such as protein or alcohol, which are characterized by a lower R.Q. than that of sugar.

R.Q. values of less than 1.0 obtained in the presence of abundant glucose may be attributed to the incomplete oxidation of glucose to form appreciable quantities of organic acids or other substances which would themselves on oxidation yield R.Q. values greater than 1.0.

The data of table 2 show that small non-legume roots, like small legume roots, are usually deficient in available energy supply. An average of all of the data in this table shows that the addition of glucose to non-legume roots produced an increase of about 40 per cent in  $QO_2$ . Such a low  $QO_2$  in the absence of added sugar, together with an R.Q. of less than 1.0, is strongly indicative of true endogenous respiration, that is, the oxidation of protein and cell constituents other than normal carbonaceous reserve compounds.

COMPARATIVE RATES OF RESPIRATION PER UNIT OF NITROGEN  
OF LEGUME NODULES, LEGUME ROOTS  
AND NON-LEGUME ROOTS

In tables 1 and 2 and in the preceding paper (3) are presented data showing the rates of respiration per unit of nitrogen,  $QO_2(N)$ , for various species of legume nodules, legume roots, and non-legume roots maintained under the various experimental conditions already discussed. In order to facilitate the discussion of the  $QO_2(N)$  data, a summary was made of all of these values, together with some additional similar data not reported in either of these papers, where both  $QO_2$  and  $QO_2(N)$  figures were available. The additional data included several values for medium and large sized nodules. The average values are given in table 3.

In considering these data it is well to point out that the original individual values from which the averages were calculated deal with a large number of variables, and that an equal number of determinations involving each variable was not conducted for each group of plants both in air and in pure oxygen. The average values, however, are based on a rather large number of individual values and are believed to be representative.

It has just been pointed out that in air the  $QO_2$  values for non-legume roots, legume roots, and small nodules were not markedly different but averaged near 2.2. The value for nodules, given in table

3, however, is lower than that for the root tissues, owing to inclusion of the results with some of the larger nodules in the averages. In contrast, the  $QO_2(N)$  values for non-legume roots and legume roots are three to four times greater than the value for nodules; the difference would have been reduced somewhat if only small nodules had been used. The slightly lower value for legume roots in comparison with that for non-legume roots may be attributed to the fact that the

TABLE 3  
COMPARATIVE AVERAGE  $QO_2$  AND  $QO_2(N)$  VALUES FOR LEGUME NODULES  
LEGUME ROOTS, AND NON-LEGUME ROOTS MAINTAINED  
IN AIR AND PURE OXYGEN

MATERIAL	$QO_2$	RELATIVE VALUES	$QO_2(N)$	RELATIVE VALUES
AIR				
Non-legume roots.....	2.28	(100)	114.8	(100)
Legume roots.....	2.12	93	92.8	81
Legume nodules.....	1.85	81	27.8	24
OXYGEN (100%)				
Non-legume roots.....	2.39	105	134.2	117
Legume roots.....	2.88	126	139.7	122
Legume nodules.....	4.98	218	73.3	64

legume roots were frequently slightly larger than the non-legume roots.

In pure oxygen the  $QO_2$  of nodules was on the average nearly twice as great as that for roots. In contrast, the average  $QO_2(N)$  value for nodules was only slightly more than half as large as for root tissues. On a unit nitrogen basis, therefore, root tissues are considerably more active even in pure oxygen than are nodule tissues and much more so under ordinary atmospheric conditions. It should be borne in mind, however, that in the previous paper (3) it was shown that although the rate of nodule respiration increases with oxygen pressure, the maximum rate was apparently (3, fig. 2) not reached even

with small nodules maintained in pure oxygen. In view of this finding, and the fact that the average values reported in table 3 included numerous determinations with medium or larger sized nodules, it seems evident that if nodule tissues could be given all the oxygen that they could consume, the difference between the  $Q_{O_2}(N)$  values of nodule and root tissues would be appreciably less. Nevertheless there does seem to be a real difference between small roots and nodules in the rate of respiration per unit of nitrogen in the tissues. This fact might be interpreted as indicating that a greater portion of the nitrogen of the nodules is in storage or translocatory materials than is the case in small roots. It is reasonable to expect this situation, of course, since the nodule cells contain an abundance of bacteria, a large portion of which seem to be inactive, and are also constantly supplying the host plant with soluble forms of nitrogen.

### Discussion

These experiments show that there is, on the average, little or no difference between the rates of respiration of small legume and small non-legume roots. Frequently roots of legumes do show higher rates of respiration than those of non-legumes, but the contrary is also true. Legume nodules ordinarily possess the capacity in the presence of pure oxygen to respire about twice as fast as a given weight of root tissue; but, because of the limited oxygen supply inside, their rate of respiration under ordinary atmospheric conditions is usually not much, if any, greater than that of roots. In the case of nodules having diameters greater than about 2 or 3 mm., the  $Q_{O_2}$  is commonly lower than for root tissues. These findings do not necessarily disagree with those respiration results obtained in field experiments that show higher rates of root respiration for legumes than for non-legumes per unit of field area. Legumes, especially perennials, commonly have larger root masses than non-legumes, especially the small grain crops frequently studied. Furthermore, soils evolve varying quantities of  $CO_2$ , and this fact tends to complicate the interpretation of data obtained in the field.

The facts presented here, together with the respiration data of the preceding paper, show that in this respect nodule behavior is in general similar to that of plant root cells and resting bacteria (or

both) and not to that of actively growing rhizobia. HOOVER and ALLISON (5, 2) have shown that very actively growing rhizobia have  $QO_2$  values of 30-40, whereas truly resting rhizobia have  $QO_2$  values of 6-8 when supplied with adequate sugar. In the absence of substrate the endogenous respiration of the bacteria may be near 1-3. In the experiments reported here a  $QO_2$  value of about 9 represented the maximum observed with nodules, and that rate occurred only in pure oxygen. The average  $QO_2$  value of 5 in pure oxygen is therefore near the value that might be expected on the assumption that the nodule consists of plant cells filled with resting bacteria. The lower  $QO_2$  value for nodules maintained in air is of practical interest, but in attempting to determine the condition of the bacteria in the nodules it is best to put more weight upon the pure oxygen results, since the low  $QO_2$  values in air are due largely to the failure of oxygen to penetrate sufficiently rapidly to meet their needs. Hence such low values do not necessarily indicate bacteria incapable of normal activity. The conception of the nodule as a place where large amounts of carbohydrates are oxidized per unit of tissue is therefore incorrect. Instead, the data are in harmony with the idea, borne out by numerous morphological studies, that the nodule consists of plant cells filled largely with bacteroids that are comparatively inactive, particularly under ordinary atmospheric conditions. But the data do not definitely exclude the possibility that a considerable portion (10 per cent or more) of the bacteria may be growing fairly rapidly, since even such active bacteria may have comparatively low  $QO_2$  values (15-25).

In a previous paper from this laboratory, ALLISON (1) attempted, on the basis of the meager respiration data then available, to estimate the carbohydrate consumption in the nodule. On the assumption that legumes respire twice as rapidly as non-legumes, owing to the bacteria in the nodules, it was calculated that "the bacterial requirements for respiration and nitrogen fixation are probably not greater than 3 to 6 per cent of the total carbohydrate photosynthesized under good growing conditions." It was further stated that this estimate was believed to be high and that the host itself consumes most of the carbohydrate. On the basis of the present data showing that under natural field conditions legume nodules with

their bacteria respire at nearly the same rate as legume and non-legume roots, and not twice as fast, we can say definitely that the carbohydrate oxidized by the bacteria in nodules is indeed a small portion of the total photosynthesized. The bacterial respiration in the nodule is not much greater than that of the equivalent weight of the host plant tissues. These findings are in harmony with practical observations to the effect that inoculated legumes, even under inadequate light conditions, do not appear to suffer much, if any, more from insufficient carbohydrate than do uninoculated legumes supplied fixed nitrogen.

### Summary

The results of respiration studies with detached nodules, legume roots, and non-legume roots from several species of plants, carried out according to standard manometric procedures, are as follows:

1. Rates of respiration of legume nodules and small legume and non-legume roots maintained in a nutrient solution in air averaged nearly the same, the  $QO_2$  values being about 2.2. Rates of respiration of the roots of various species of plants showed no marked differences for roots of comparable age, size, and condition.

2. Respiratory quotients of small legume and non-legume roots maintained in air were usually slightly less than 1.0 and hence were definitely lower than for nodules, although large roots may also show values considerably above 1.0 because of slow oxygen penetration.

3. Rates of respiration per unit of dry matter of legume nodules in pure oxygen were more than twice as great as in air, whereas values for the small legume and non-legume roots were usually not markedly affected by the additional oxygen. Nodules therefore have an inherent capacity for respiring at a more rapid rate than root tissues, provided the inner cells can get the necessary oxygen.

4. Small legume and non-legume roots are more deficient in available energy supply than are nodules, a mean increase in  $QO_2$  of about 40 per cent due to glucose additions to roots being obtained, compared with 12-14 per cent with nodules.

5. Rates of respiration per unit of nitrogen of legume and non-legume roots maintained in air were three to four times as great as for nodules. Even in pure oxygen the  $QO_2(N)$  values of roots were on the average about twice as great as for nodules.

6. These data are in harmony with the idea, so frequently suggested by morphological studies, that the nodule consists of plant cells largely filled with comparatively inactive bacteria. The bacteria oxidize only a very small portion of the total carbohydrate photosynthesized by the host plant.

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## CYTOLOGICAL STUDIES OF LILIUM TIGRINUM

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 509

JONATHAN J. WESTFALL

(WITH SIXTY-NINE FIGURES)

### Introduction

Because it is the only species of *Lilium* reported to possess both diploid and triploid forms, *L. tigrinum* Ker-Gawl. is of special interest. Early investigators reported only the diploid form, but descriptions and figures given in several papers indicate that some of the plants reported as diploids were in reality triploids.

COULTER, CHAMBERLAIN, and SCHAFFNER (13), BELLING (8), and SIANG (36) reported 12 pairs of synaptic mates in the meiotic divisions of the microsporocytes. More recently TAKENAKA and NAGAMATSU (42) reported that this species possessed 36 somatic chromosomes. SATO (32) confirmed this work on the single-flowered variety, and reported that *L. tigrinum* var. *flore-pleno* also has 36 chromosomes. TAKENAKA (41) observed lagging, fragmentation, and other irregularities in the meiotic divisions of the microsporocytes of *L. tigrinum*. In the following year SASS (30) found similar irregularities. MATHER (21) extended the studies to include the additional *tigrinum* varieties *Fortunae giganteum* and *splendens*. Both were found to possess 36 chromosomes. CHANDLER, PORTERFIELD, and STOUT (11) studied chromosome morphology and microsporogenesis in both the diploid and triploid forms. The existence of this self-fertile species of *Lilium*, which proved to be of the diploid type, had previously been reported by PRESTON (25). Chromosome counts of a number of seedlings of the triploid *L. tigrinum* were reported by SATO (33).

Following the account of development of the megagametophyte of *Fritillaria persica*, in which BAMBACIONI (1) described the process wherein four nuclear divisions intervene between the archesporial cell and the egg instead of three, as was formerly supposed, other members of the Liliaceae were examined or reexamined in rapid succession.

BAMBACIONI and GIOMBINI (2) reported a similar process in *Tulipa gesneriana*. This was followed by the work of BAMBACIONI-MAZETTI (3) on *Lilium bulbiferum*, *L. candidum*, and *Tulipa praecox*. Both *T. praecox* and *T. gesneriana* have since been found to be triploids (15). Although her report of the development of the megagametophyte of these two species emphasizes the division and orientation of the nuclei rather than the determination of the exact numbers of chromosomes, some of the illustrative figures included show lagging chromosomes and micronuclei similar to those reported for other triploid plants.

COOPER (12) described the method of development of the megagametophyte in *Lilium henryi* and examined preparations of *L. speciosum*, *L. philadelphicum*, *L. longiflorum* var. *eximium*, *L. regale*, *L. auratum* var. *platyphyllum*, *L. elegans*, *L. martagon*, *L. pardalinum*, and *L. philippinense*. Later SANTOS (29) described the complete process in *L. philippinense*. In all species of *Lilium*, *Fritillaria*, and *Tulipa* examined it was found that in the course of the third nuclear division three of the four nuclei formed as the result of the second meiotic division migrate to the chalazal end of the megagametophyte, and there the three spindles fuse and undergo mitosis. At the same time the lone nucleus at the micropylar end also divides mitotically, so that a second 4-nucleate stage results. Instead of all nuclei being haploid, however, as in the first 4-nucleate stage, the two micropylar nuclei are at the later stage haploid, but the two chalazal nuclei are triploid. One further mitotic nuclear division occurs, resulting in the formation of four nuclei at each end of the megagametophyte. One nucleus from each end, the polar nuclei, move toward the center and unite, the nucleus from the chalazal end contributing  $3n$  chromosomes and the nucleus from the micropylar end bringing the total count of the fusion nucleus to  $4n$  in the diploid plants. In the process of fertilization one of the male gametes unites with the egg at the micropylar end to form the zygote, while the other unites with the polar nucleus to form the primary endosperm nucleus. This type of megagametophyte development has been designated by SCHNARF (35) as the "*Fritillaria* type," to avoid confusion with the "*Lilium* type" in the older sense.

In other members of the Liliaceae microsporogenesis has been

studied in the triploid forms of *Hyacinthus* by BELLING (6); *Hosta* by YASUI (45); *Hemerocallis* by BELLING (7); *Tulipa* by DARLINGTON (14), NEWTON (24), and WOODS (44); and *Allium* by LEVAN (20).

Among the limited number of reports on the development of the megagametophyte in triploids are those of MORINAGA and FUKUSHIMA (22) on *Oryza* and of SATINA and BLAKESLEE (31) on *Datura stramonium*.

If assortment of chromosomes and distribution of nuclei were the same in the development of the female gametophyte as in the male, studies of both would not be necessary; but the chromosome numbers of the progeny of triploids may be different, depending on whether the triploid is the pollen parent or the seed parent or both. As a general rule extra chromosomes are more often transmitted to the progeny by the female gametes than by the male (14), possibly because of complete failure of growth of the abnormal pollen grains or their failure to compete with pollen grains having the haploid set of chromosomes. Other features, as chromosome lagging, fragmentation, and random assortment—characteristic of autotriploids—as well as the type of division and arrangement of nuclei, make *Lilium tigrinum* particularly interesting for cytological study.

Since SCHAFFNER (34), SASS (30), TAKENAKA (41), and CHANDLER, PORTERFIELD, and STOUT (11) previously reported studies of microsporogenesis in *L. tigrinum*, the present study devotes attention to megasporogenesis and development of the megagametophyte, but makes frequent reference to observations on microsporogenesis.

#### Material and methods

Anthers and ovaries of *L. tigrinum* var. *splendens* were gathered from plants grown in the experimental garden at the University of Chicago and at Wychwood on Lake Geneva, Wisconsin, during late July and the first two weeks in August of the summer of 1938. The bulbs were originally obtained from Wayside Gardens, Mentor, Ohio.

The iron-aceto-carmin smear method was used in making preliminary studies and in determining the proper stages for fixation of the anthers. In most instances the first meiotic division was found to take place when the buds were about  $1\frac{1}{4}$  inches in length, or

about 2-3 days before anthesis. Anther material was fixed at different times during the day over a period of 3 weeks. Ovaries were fixed one day before anthesis and continued at 24-hour intervals until 120 hours after anthesis. The ovaries were cut either longitudinally or transversely to reduce the distance the fixatives had to penetrate. Some of the ovules were dissected out before being fixed.

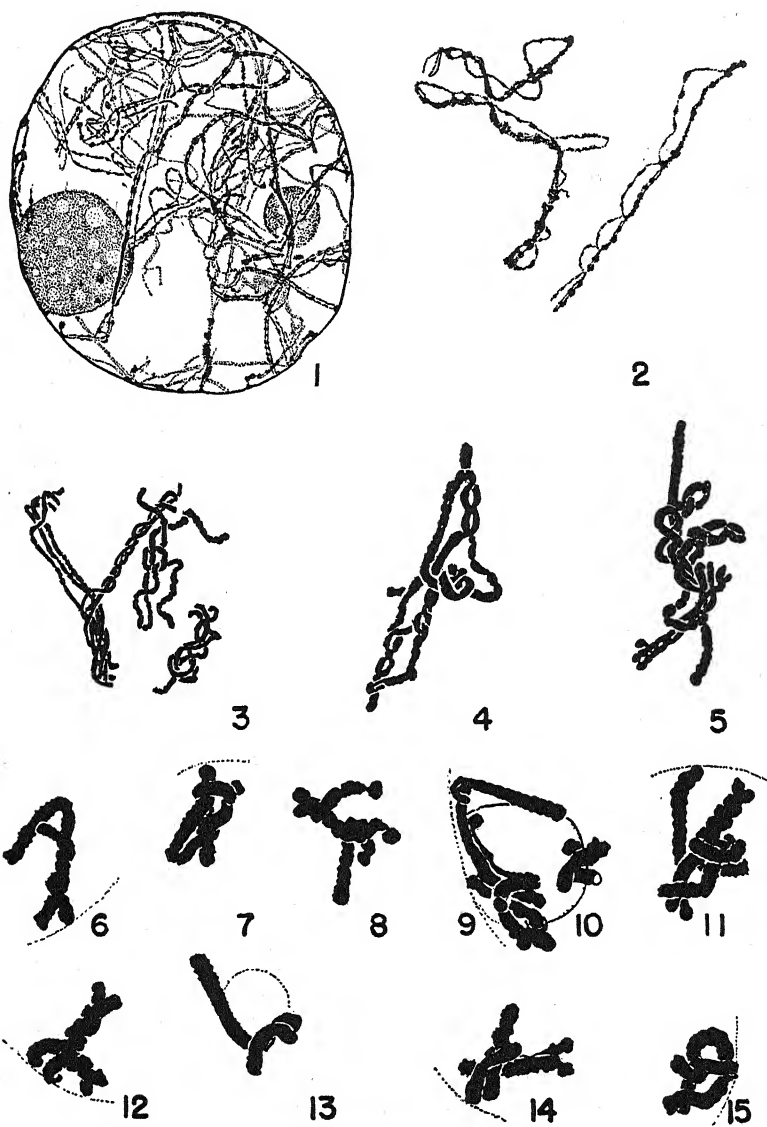
Smears of the anthers were fixed in Belling's modification of Navashin's and in Carnoy's solutions. Other anthers and the ovaries were allowed to remain in Carnoy's for about 80 seconds, after which they were placed in Navashin's solution: This treatment, as well as using Flemming's or Navashin's alone, gave good fixations. The butyl-alcohol paraffin method of imbedding was used, and sections were cut chiefly at 25-30  $\mu$ , although some sections for critical studies of the chromosomes were cut as thin as 6  $\mu$ . Either Heidenhain's iron-alum haematoxylin or the crystal violet-iodine method was used in staining all paraffin sections.

Observations were made with either a 10 $\times$  or a 15 $\times$  compensating ocular and a 100 $\times$  fluorite objective. Drawings were made at table level with a camera lucida.

### Observations

Critical examination of stages in the development of *L. tigrinum* var. *splendens* indicates that with few exceptions development follows the general plan reported for other species of *Lilium*, *Fritillaria*, and *Tulipa*. In the present paper no attempt is made to reiterate the story of division and orientation of nuclei or to illustrate all stages in developmental sequence. Only those phases in which *L. tigrinum* has been found to differ from other reported species are given particular attention.

In the early prophase of both the megasporocytes and the microsporocytes, chromosomes occur as single or as paired threads. Pairing, first evident at zygotene, is even more apparent at pachytene, when the threads have increased in chromaticity and have contracted somewhat longitudinally. At this stage (fig. 1) two of the synapsed chromosomes are closely associated and coiled throughout long segments, while the third apparently homologous chromosome



FIGS. 1-15.—Prophases and diakinesis in megasporocytes: fig. 1, nucleus at pachytene; fig. 2, late pachytene threads showing type of chromosome association in detail; figs. 3-5, diplotene and early diakinesis showing entangled trivalent groups; figs. 6-15, trivalent associations of single nucleus at diakinesis.

is in contact with the two at irregular intervals (fig. 2). In most instances the two closely synapsed chromosomes appear to have contracted longitudinally more rapidly after pairing than did the third member. Contraction does not seem to be dependent on pairing but is initiated sooner and progresses more rapidly when pairing has taken place. Differential contraction in some instances causes apparent failure of correspondence in chromomere arrangement in homologous chromosomes. At diplotene and early diakinesis (figs. 3-5) the chromatids of each chromosome are plainly visible, and frequently the members of two or more trivalent associations are

TABLE 1  
FREQUENCY OF TRIVALENT, BIVALENT, AND UNIVALENT CHROMOSOME ASSOCIATIONS AT DIAKINESIS AND METAPHASE I;  
DATA FOR 500 MICROSPOROCYTES

ASSOCIATIONS	FREQUENCY	PERCENTAGE FREQUENCY
12 trivalents.....	293	58.6
11 trivalents, 1 bivalent, and 1 univalent.....	155	31.0
10 trivalents, 2 bivalents, and 2 univalents.....	39	7.6
9 trivalents, 3 bivalents, and 3 univalents.....	11	2.2
8 trivalents, 4 bivalents, and 4 univalents.....	2	0.6
Less than 8 trivalents.....	0	0.0
Mean no. of trivalents for entire sample.....	.....	11.45

entangled so as to produce an apparent secondary association. This is interpreted as a result of chance arrangement of chromosomes preceding pairing, so that true or false interlocking results, rather than as interchange of parts between nonhomologous chromosomes. At diakinesis a single nucleus generally contains three nucleoli of different sizes, although more or less than this number may be present.

Preliminary studies of chromosome associations in the microsporocytes and megasporocytes at diakinesis show no significant differences in the degree of frequency of trivalents, bivalents, and univalents in the two types of cells. Since it is easier to make counts from smear preparations of the microsporocytes, they were used in determining the frequency of different types of associations.

Chromosomes of two or more trivalent groups often remain inter-

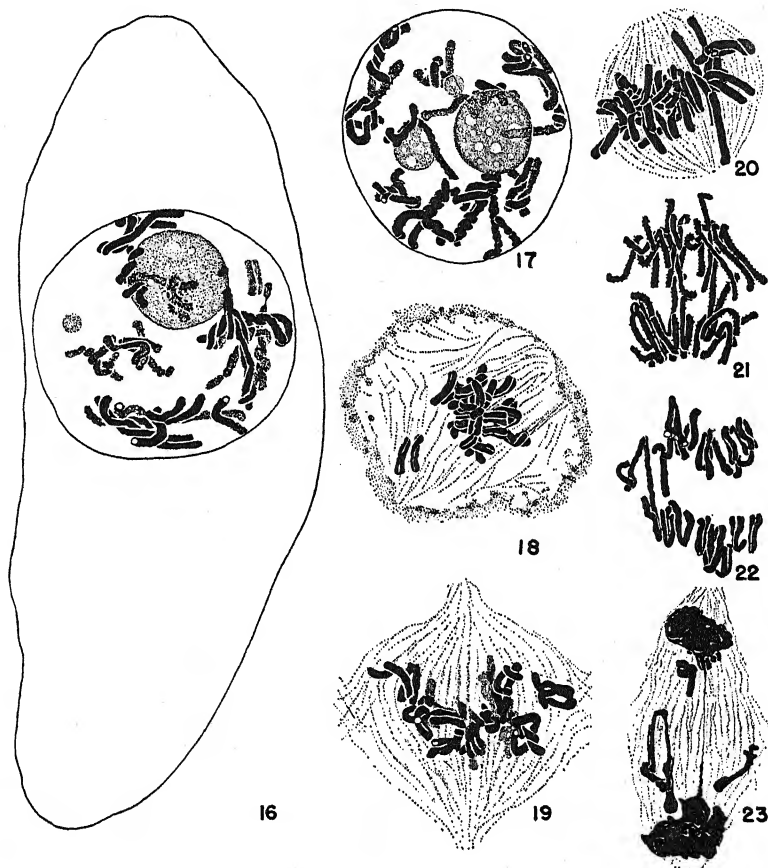
meshed throughout diakinesis (figs. 9, 11), but 12 separate trivalent associations occur more frequently.

Trivalents shown in figures 5-15 were drawn from a single nucleus, and in all cases at least one chromosome of each association was in contact with the nuclear membrane. The trivalents are oriented in such a manner that each association, with the exception of those entangled, is well separated from the next, as if repelled. This is not so apparent in single-plane drawings as it is in the material from which they were drawn. In figure 16 the chromosomes are grouped as 11 trivalents, 1 bivalent, and 1 univalent. All chromosomes are grouped as 12 trivalent associations in figure 17. Two univalents are shown apart from the compactly grouped trivalent and bivalent mass in figure 18. Dissociation of some of the trivalents is shown at early metaphase in figure 19.

#### FIRST MEIOTIC DIVISION

The arrangement of chromosomes at the equatorial plane during metaphase of the first meiotic division seems to be without unusual features (figs. 19, 20). Only occasionally is a univalent left outside the plane toward the poles. The first meiotic division is generally characterized by complete disjunction of all members of trivalent and bivalent associations, although there are two notable exceptions to this general rule. First, chromatids occasionally fail to disjoin at anaphase and tend to adhere near their ends, which are bent out at right angles to the axes of the two chromatids. This type of behavior is shown in the center of figure 21 and in the first of the three bridges of figure 53. The chromatids may eventually disjoin, or non-disjunction may persist in the microsporocytes until after the 2-celled stage. The plainly visible ends of the bridging chromatids and the absence of acentric fragments distinguish this from a more common type of bridge shown to the right of center in figure 21 and to the left of center in figure 22. In figure 23, in addition to four lagging chromosomes between the two organizing telophase nuclei, there is a fragment and an attenuated chromatid bridge which appears to be stretched almost to the breaking point. The frequency of such bridges appears to be about the same in microsporogenesis and megasporogenesis. Of 603 microsporocytes examined, seventy,

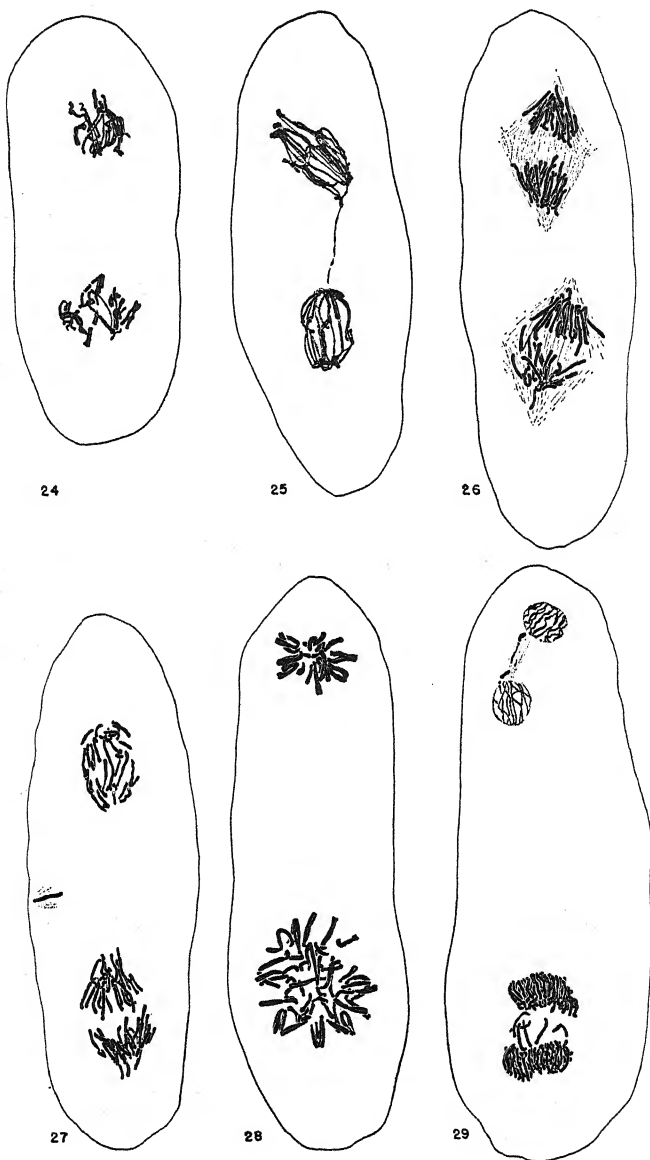
or 11.6 per cent, showed the presence of anaphase chromatid bridges. These bridges occasionally persist even after the formation of the



FIGS. 16-23.—Fig. 16, megasporocyte at diakinesis showing 11 trivalents, 11 bivalents, and 1 univalent; fig. 17, nucleus at diakinesis showing trivalent associations and nucleolar attachments; figs. 18-20, metaphase of first meiotic division; figs. 21, 22, anaphase showing chromatid bridges; fig. 23, telophase showing lagging chromosomes and attenuated chromatid bridge.

division figure of the second meiotic division (fig. 25). In figure 30 the two spindles of the second meiotic division are seen unusually close together, still joined by a persisting chromatid connection.

The first meiotic division of the microsporocyte differs from that



FIGS. 24-29.—Fig. 24, metaphase of second meiotic division; fig. 25, early anaphase figures of second meiotic division; figs. 26, 27, anaphase of second meiotic division; fig. 28, metaphase of third nuclear division; fig. 29, 4-nucleate stage following third nuclear division, showing lagging at both micropylar and chalazal ends.

of the megasporocyte in that in the former a cell wall is formed between the two nuclei before the second division. As this cell wall forms it may cut in two any bridges persisting after the first division. More often, however, the bridge prevents the formation of the cell wall at the point through which it passes in connecting the two nuclei. Prevention of cell wall formation is regarded not so much as an inhibitive action on the part of the chromatin material as a mere replacement of the wall-forming cytoplasm at the point where the bridge passes through.

Figure 49 of the division of the microsporocyte is comparable with figure 22 of the megasporocyte. In both figures one of each pair of attached chromatids has completely disjoined, while the other, with the chromatid from which it failed to separate, forms a taut bridge between the separating masses of chromosomes. A sharply bent fragment is shown near the midpoint of each bridge. In addition to the chromosome fragments, lagging chromosomes also occur (figs. 49-51). Figures 49-62 show chromatid bridges and acentric fragments. In the development of the megagametophyte attenuated chromatid bridges are generally broken and may be absorbed in the cytoplasm. Fragments and lagging chromosomes may be absorbed, or they may persist as micronuclei or as granules.

Accurate determination of chromosome distribution at the first and second meiotic divisions was impossible except in a limited number of very favorably oriented cells which showed no lagging. In figure 21 the chromosomes, including those making up the bridge, are evenly divided, 18-18. In figure 22 the distribution is 19-17, including the chromosomes forming the bridge. Counts were made of 27 megasporocytes at the first meiotic division, and the chromosome numbers of the daughter nuclei were found to vary from 12 to 22, excepting 13 and 20. The mean for the distribution was 17.1. These figures are only approximate, because in the division of the megasporocyte no incoming cell wall marks off the cytoplasm into two compartments, and it is therefore impossible to say with which of the daughter nuclei, if with either, the lagging chromosomes should be counted. If they are credited with neither nucleus, or if only figures showing no lagging are counted, the story of chromosome distribution is still incomplete.

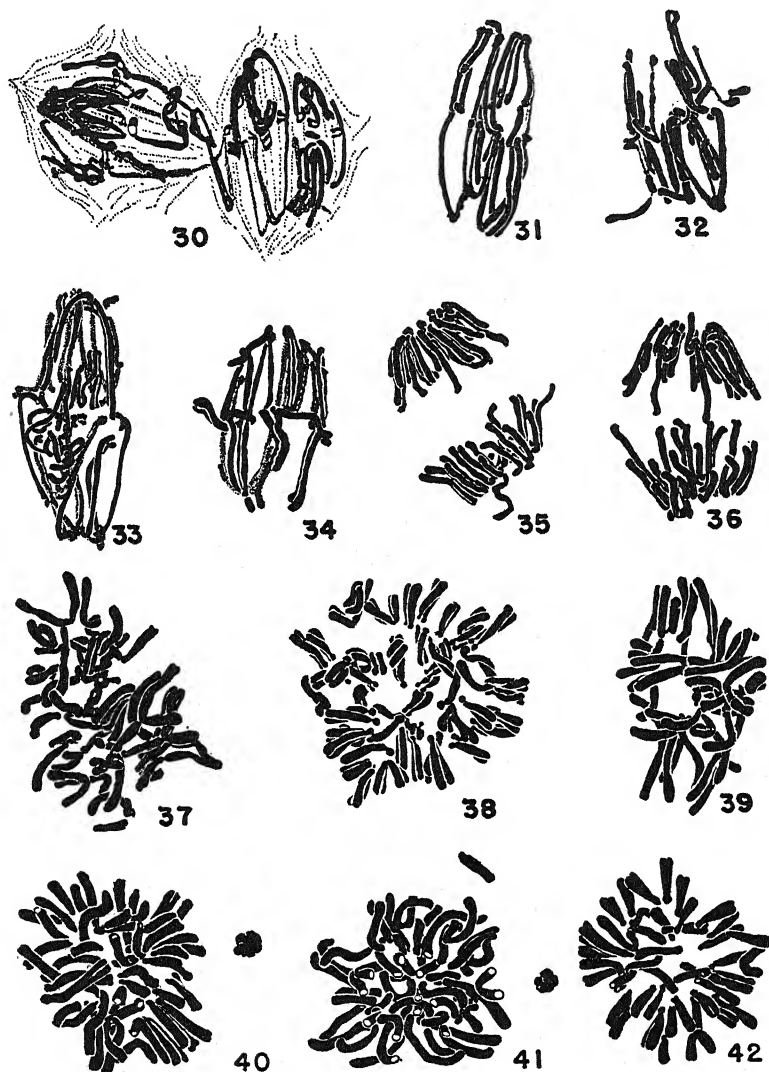
At metaphase of the second meiotic division (figs. 24, 25) few accurate counts could be made because of the manner in which the chromosomes seem to adhere to one another. Further difficulty in making counts was caused by the four chromatids of two adjacent chromosomes with subterminal constrictions being so easily confused with two chromatids of a single chromosome with submedian spindle attachment constriction. With the data from figures that could be counted, however, and from counts at later stages, it seems probable that at the first meiotic division one set of 12 chromosomes goes to one pole and 12 to the other, while the members of the third set are randomly distributed between the two. Of 603 microsporocytes examined, lagging was detected in 47.9 per cent.

#### SECOND MEIOTIC DIVISION

In the second meiotic division also there is both lagging and bridging. In the lower spindle of figure 26 and in the upper spindle of figure 27 bridging is shown. Approximately midway between the two spindles of figure 27 is shown a lagging chromosome or large fragment in contact with a small accessory spindle. The second meiotic division of the microsporocyte is shown in figure 63. Both bridging and lagging are shown in the lower cell of the two. Typical configurations at metaphase of the second meiotic division are shown in figures 31, 32, and 34. The upper spindle of figure 26 and the lower of figure 27 show the second meiotic division as it more commonly occurs, without bridging and lagging.

In the absence of lagging and bridging the second meiotic division is numerically equational. The effects of lagging, bridging, and fragmentation cause differences in chromosome numbers as great as five in the two nuclei formed as the result of division of a single nucleus. Counts were made of five megasporocytes at the second meiotic division. These limited data are inadequate for drawing conclusions as to the most common assortments, but they serve to illustrate some of the effects of lagging and fragmentation (table 2).

The chromatids, when separated precociously in the first meiotic division, do not divide again in the second. It is possible that owing to unequal distribution of chromosomes the four nuclei formed as a result of the second meiotic division may have different chromosome



FIGS. 30-42.—Fig. 30, division figures of second meiotic division in megasporocyte connected by persisting chromatid bridge; figs. 31-34, late metaphase of second meiotic division; figs. 35, 36, second meiotic division at anaphase; figs. 37-42, chalazal nuclei showing differences in chromosome numbers.

numbers, although this is not usually the case. In the megasporocytes the lagging elements generally roll up in compact masses in the cytoplasm and finally disintegrate or form micronuclei. In the microsporocytes the behavior is much the same except that cell walls may form, splitting off the micronuclei and surrounding cytoplasm to form microcytes. Fragments may persist in the cytoplasm until after the microspores are fully formed.

TABLE 2  
DISTRIBUTION OF CHROMOSOMES IN 5 MEGASPOROCYTES AT  
SECOND MEIOTIC DIVISION

FIRST SPINDLE			SECOND SPINDLE		
1ST NUCLEUS	LAGGING	2D NUCLEUS	1ST NUCLEUS	LAGGING	2D NUCLEUS
19	.....	19	17	1	16
20	.....	20	16	.....	16
22	.....	22	13	.....	13
17	.....	17	12	4	19
18	.....	18	18	.....	18

### THIRD AND FOURTH NUCLEAR DIVISIONS

The somatic number of chromosomes is 36 ( $3n$ ), and if each daughter nucleus should receive equal numbers, the nuclei at the first 4-nucleate stage following the second meiotic division would have 18 chromosomes each. Fusion of three of these nuclei at the chalazal end of the megagametophyte would lead to the formation of a nucleus having 54 chromosomes. The constitution of this chalazal nucleus would be, not  $3n$  as in the diploid, but  $4\frac{1}{2}n$ . These theoretical numbers might be found only in those cases in which there is an equal assortment of chromosomes at both first and second meiotic divisions and no loss through lagging and fragmentation. Counts made at the metaphases of the third nuclear division (fig. 28) showed that the chromosome numbers at both chalazal and micropylar ends vary markedly from the theoretical values. The calculated distribution from random assortment was found by expansion of the binomial. As is shown in tables 3 and 4, both chalazal and micropylar nuclei near or at the upper and lower limits of distribution occur

more frequently than would be expected from random assortment, and correspondingly there are less nuclei with chromosome numbers near the calculated mean than would be expected. If there were no loss of chromosomes in the nuclear divisions of the megagametophyte, the expected number of chromosomes in the chalazal nucleus at the metaphase of the third division might be determined by

TABLE 3

CHROMOSOME NUMBERS IN 150 NUCLEI AT MICROPYLAR END OF MEGAGAMETOPHYTE AT METAPHASE OF FOURTH NUCLEAR DIVISION

CHROMOSOME NO.	FREQUENCY	PERCENTAGE		PERCENTAGE $\frac{\text{OBSERVED}}{\text{CALCULATED}}$
		OBSERVED	CALCULATED ON RANDOM ASSORTMENT	
3.....	1	0.66	0.00	.....
12.....	6	4.00	0.02	166.60
13.....	13	8.66	0.31	27.93
14.....	19	12.66	1.61	7.86
15.....	26	17.33	5.37	3.22
16.....	28	18.66	12.08	1.54
17.....	25	16.66	19.33	0.86
18.....	11	7.33	22.60	0.32
19.....	7	4.66	19.33	0.24
20.....	2	1.33	12.08	0.10
21.....	2	1.33	5.31	0.24
22.....	5	3.33	1.61	2.06
23.....	0	0.00	0.31	.....
24.....	3	2.00	0.02	83.33
31.....	1	0.66	0.00	.....
38.....	1	0.66	0.00	.....

Mean chromosome no. of observed nuclei..... 16.82

Mean calculated from random assortment..... 18.00

counting the number in the micropylar nucleus and applying the formula,  $72 - M = C$ , where  $M$  is the number of chromosomes in the micropylar nucleus and  $C$  is the number in the chalazal nucleus. At the metaphase of the fourth division the number of chromosomes would be approximately twice that of the third.

A comparison of the observed numbers of chromosomes in the chalazal nuclei with those calculated from random assortment gave the numbers lacking owing to loss through lagging and fragmenta-

tion in the previous divisions. The number lacking,  $L$ , may be obtained for the metaphase of the third nuclear division by the formula,  $72 - (M + C) = L$ . In a study of 28 megagametophytes in which accurate counts could be made at both micropylar and chalazal ends, it was found that 42.85 per cent of the nuclei showed loss of chromosomes, averaging 8.2 chromosomes, or 11.11 per cent loss per cell. This does not mean that this number of chromosomes was actually left out of the nuclei, but that the number lacking owing to loss is as given. Loss of chromosomes in the first division would appear as a deficiency twice as great at metaphase of the third division, and as a deficiency four times as great at metaphase of the fourth division.

Counts were made of chromosomes in 55 micropylar nuclei at metaphase of the third nuclear division and of 150 at the metaphase of the fourth division. In general the distribution was similar in the two stages, the principal difference being a slight shift toward the lower chromosome numbers in the later stage, as shown by a mean of 16.82 chromosomes at metaphase of the third division as compared with 16.25 for the fourth. The difference is probably due to loss of chromosomes during the third division. In all the megagametophytes examined, with the exception of two, the numbers of chromosomes in the micropylar nuclei ranged from 12 to 24 (table 3, fig. 68). In one of these exceptions one nucleus at the micropylar end had 3 chromosomes and the other had 31. In the other case a restitution nucleus possessing 36 chromosomes apparently was formed, as indicated by the presence of two chalazal nuclei and only one micropylar nucleus.

Chromosome counts were made of 47 chalazal nuclei at metaphase of the third nuclear division and of 41 at metaphase of the fourth division. At metaphase of the third division chromosome numbers ranged from 37 to 60, excepting 39 (table 4, fig. 69). At metaphase of the fourth division the range was from 33 to 60, excepting 34 and 35. The mean number per nucleus at metaphase of the third division was 50.04 as compared with 49.85 for the fourth. Except for this slight difference, the distributions of chromosome numbers were similar in the two stages.

If the third and fourth nuclear divisions in the development of the

megagametophyte were mitotic and equational, as has been reported for other species of *Lilium*, the female gametes of the triploid *L. tigrinum* should receive the number of chromosomes occurring in

TABLE 4

CHROMOSOME NUMBERS IN 47 CHALAZAL NUCLEI OF MEGAGAMETOPHYTE  
AT METAPHASE OF THIRD NUCLEAR DIVISION

CHROMOSOME NO.	FREQUENCY	PERCENTAGE		PERCENTAGE $\frac{\text{OBSERVED}}{\text{CALCULATED}}$
		OBSERVED	CALCULATED ON RANDOM ASSORTMENT	
37.....	1	2.12	0.00	.....
38.....	2	4.25	0.00	.....
39.....	0	0.00	0.00	.....
40.....	1	2.12	0.00	.....
41.....	1	2.12	0.00	.....
42.....	2	4.25	0.00	.....
43.....	1	2.12	0.00	.....
44.....	2	4.25	0.00	.....
45.....	1	2.12	0.00	.....
46.....	1	2.12	0.00	.....
47.....	3	6.38	0.00	.....
48.....	1	2.12	0.02	88.33
49.....	3	6.38	0.31	20.58
50.....	3	6.38	1.61	3.96
51.....	2	4.25	5.37	0.79
52.....	1	2.12	12.08	0.17
53.....	3	6.38	19.33	0.33
54.....	6	12.72	22.60	0.56
55.....	4	8.51	19.33	0.44
56.....	3	6.38	12.08	0.52
57.....	3	6.38	5.37	1.18
58.....	1	2.12	1.61	1.31
59.....	1	2.12	0.31	6.83
60.....	1	2.12	0.02	88.33

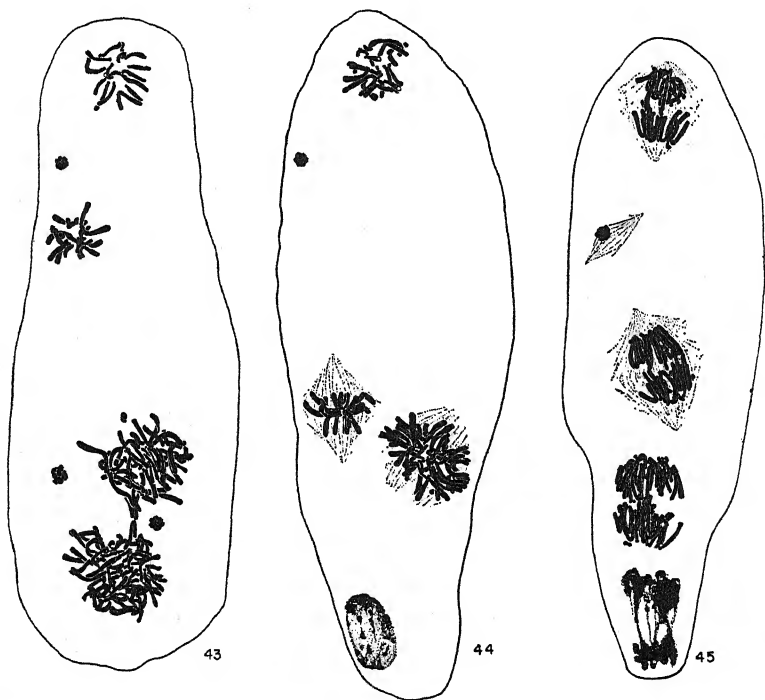
Mean chromosome no. of observed nuclei..... 50.04

Mean calculated from random assortment..... 54.00

the micropylar nuclei preceding the third nuclear division, and likewise the chalazal nuclei at this stage should have the same number of chromosomes as are later contributed to the polar nuclei which subsequently function in the formation of the primary endosperm nucleus. As shown in figure 29, however, lagging in the third and fourth nuclear divisions of both micropylar and chalazal nuclei may

lower the chromosome numbers of nuclei formed by the divisions following meiosis. The nuclei at the micropylar and chalazal ends of figure 29 actually occurred in different cells, but have been placed together for economy of space.

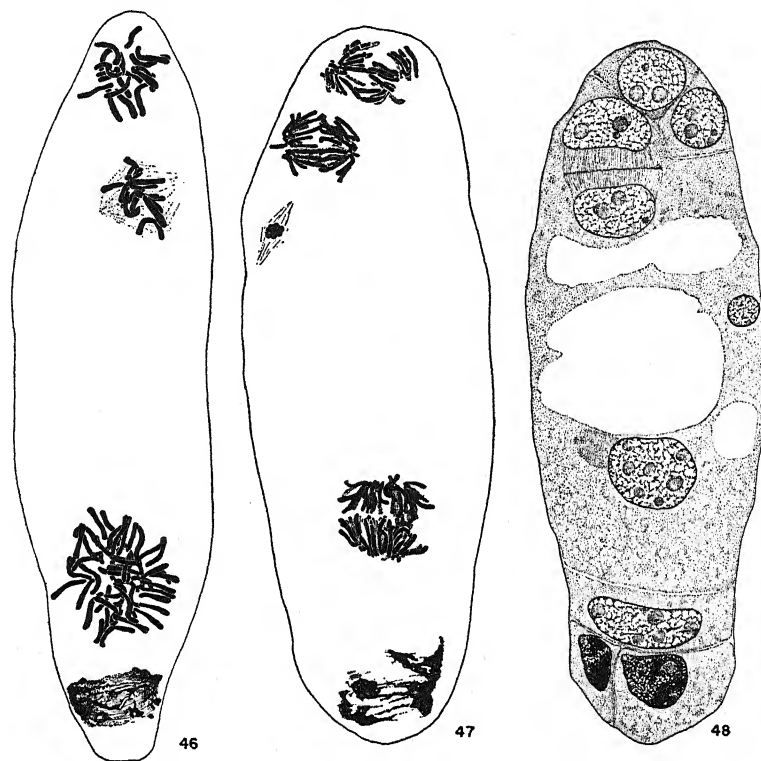
In figures 43 and 44, representing metaphase stages of the fourth nuclear division, compactly knotted lagging chromosomes are



FIGS. 43-45.—Fig. 43, metaphase in fourth nuclear division in development of megagametophyte, showing lagging chromosomes in cytoplasm; fig. 44, metaphase of fourth nuclear division showing widely separated micropylar nuclei; fig. 45, anaphase of fourth nuclear division with micropylar nuclei widely separated.

shown. It is likely that some of these chromatic masses contain more than one chromosome. In figure 45 a chromatic body is shown with a small spindle organized about it. In none of these small spindles have divisions been observed. In figure 48 is a micronucleus which was probably organized from lagging chromosomes lost from the other nuclei. At metaphase of the fourth division the usual

orientation of the four nuclei is as shown in figure 46, with two small nuclei at the micropylar end and two larger ones at the chalazal end. This orientation is generally maintained effectively by the formation of one or more large vacuoles between the two sets of



FIGS. 46-48.—Fig. 46, metaphase of fourth nuclear division as it usually appears; fig. 47, anaphase of fourth nuclear division showing apparently amitotic division of lower chalazal nucleus and a chromatic body in the cytoplasm; fig. 48, 8-nucleate megagametophyte showing egg and two synergids at micropylar end, two polar nuclei and micronucleus toward center, and three antipodal cells at chalazal end.

two nuclei each. Frequently, however, vacuoles may form between the two micropylar nuclei, in which case one is crowded away from the other and forced down into the region of the chalazal nucleus (figs. 44, 45). Still less frequently all four nuclei may be forced by vacuole formation into either the micropylar or the chalazal end.

The lower chalazal nucleus divides apparently amitotically in the fourth division (figs. 44-47), while the upper chalazal nucleus forms a well-spaced metaphase plate (fig. 46). It was at this stage that counts were made. The 8-nucleate megagametophyte is represented in figure 46, in which a small micronucleus is also shown.

### Discussion

It has been reported that the diploid *Lilium tigrinum* sets seed readily when self-pollinated, but that the triploid form rarely does (32). STOUT (38, 39), who collected over 200 types of *L. tigrinum* which produced no seeds following self-, close-, and inter-pollination, states that the single-flowered clones of this species known previous to 1932 have been completely fruitless to cross- and self-pollination. SATO (33) reported chromosome counts of the progeny of self- and inter-pollinated plants of *L. tigrinum* and found a wide variation in chromosome numbers, ranging from 24 to 39, excepting 31. He concluded that these seedlings must have resulted from the fusion of gametes with irregular numbers of chromosomes produced by an autotriploid mother plant. From a consideration of the chromosome numbers of the progeny, he presumed the existence of gametes ranging from 12 to 27, with 12 and 14 chromosomes in nuclei occurring most frequently. He also reported the occurrence of fragments, and postulated a structural change in some of the chromosomes.

The plants used in the present investigation have been observed for a number of years and have never been seen to produce capsules following self- or inter-pollination. That sterility is due principally to self-incompatibility in the clones of the triploid *L. tigrinum* rather than to failure of formation of functional gametes is shown by the production of both capsules and viable seeds when cross-pollinated with other species, using the triploid *L. tigrinum* as seed parent in some instances and as pollen parent in others. Table 5 lists successful crosses taken from several different reports.

No cytological work has been reported on any of the *L. tigrinum* hybrids, but in view of the present investigation it seems probable that such hybrids are likely to be subject to irregularities in meiotic divisions and to show considerable variations in somatic numbers. Data given by CHANDLER, PORTERFIELD, and STOUT (11) on the

division of the microsporocyte, and additional data obtained in the present investigation on the divisions of both the microsporocytes and the megasporocytes of *L. tigrinum*, indicate that a set of 12 chromosomes passes to each pole in the first meiotic division, and that members of the third set are more or less randomly distributed

TABLE 5  
LILIUM TIGRINUM CROSSES

LILIUM SEED PARENT AND POLLEN PARENT	INVESTIGATOR AND DATE
<i>tigrinum</i> × <i>maximowiczii</i> .....	Preston (25), 1925
<i>tigrinum</i> var. <i>splendens</i> × <i>maximowiczii</i> .....	Stout (38), 1926
<i>tigrinum</i> × <i>sutchusense</i> .....	Beal (4), 1937
<i>tigrinum</i> × <i>warleyense</i> .....	Stout, 1926
<i>tigrinum</i> × <i>willimottiae</i> .....	Preston (26), 1933
<i>tigrinum</i> var. <i>splendens</i> × <i>willimottiae</i> .....	Preston, 1933
<i>tigrinum</i> × <i>davidii</i> .....	Beal, 1936
<i>tigrinum</i> × <i>amabile</i> .....	Preston, 1933
<i>tigrinum</i> × <i>elegans</i> .....	Stooke (37), 1937
<i>tigrinum</i> × <i>elegans wallacei</i> .....	Stooke, 1937
<i>tigrinum</i> × <i>pseudotigrinum</i> .....	Stout, 1926
<i>tigrinum</i> × <i>tigrimax</i> .....	Preston, 1933
<i>tigrinum</i> var. <i>splendens</i> × <i>tigrimax</i> .....	Preston, 1933
<i>tigrinum</i> var. <i>splendens</i> × <i>auratum</i> .....	Beal, 1937
<i>tigrinum</i> var. <i>splendens</i> × <i>batemanniae</i> .....	Beal, 1937
<i>tigrinum</i> var. <i>splendens</i> × <i>regale</i> .....	Beal, 1936
<i>tigrinum</i> var. <i>splendens</i> × <i>superbum</i> .....	Beal, 1936
<i>tigrinum</i> var. <i>splendens</i> × <i>leichtlinii</i> .....	Beal, 1937
<i>speciosum</i> × <i>tigrinum</i> .....	Preston, 1933
<i>bulbiferum croceum</i> × <i>tigrinum</i> var. <i>splendens</i> ..	Stooke, 1937
<i>henryii</i> × <i>tigrinum</i> .....	Preston, 1933
<i>henryii</i> × <i>tigrinum</i> var. <i>splendens</i> .....	Beal, 1936
<i>speciosum</i> × <i>tigrinum</i> .....	Preston, 1933
<i>davidii</i> × <i>tigrinum</i> (2 <i>n</i> -24) var. <i>fortuneae</i> .....	Preston, 1933
<i>maximowiczii</i> × <i>tigrinum</i> .....	Preston, 1933
<i>regale</i> × <i>tigrinum</i> .....	Preston, 1933
<i>regale</i> × <i>tigrinum</i> var. <i>splendens</i> .....	Beal, 1937
<i>superbum</i> × <i>tigrinum</i> var. <i>splendens</i> .....	Beal, 1936

between the two poles. Elimination of chromosomes causes a general shift toward the lower numbers from the distribution expected on random assortment. The result is that nuclei with *n* number of chromosomes or with *n* + 1 or *n* + 2 occur with much greater frequency than would be expected from random assortment. That loss of chromosomes through lagging is not entirely responsible for the greater than expected frequencies of nuclei with low chromosome

numbers is shown by the occurrence of many more nuclei than is expected at the upper limit of distribution. Even after having the chromosome numbers lowered by lagging and fragmentation, nuclei at or near the diploid end of the distribution occur in greater than expected frequencies (figs. 68, 69; tables 3, 4).

After allowance is made for the general shift to the left, the distribution approaches that expected from random assortment, except that fewer nuclei are found at or near the mean and more at or near the haploid and diploid limits than expected. A departure from calculated distribution similar to this has been reported for several triploids. BELLING (5) for *Canna*, DERMEN (16) for *Petunia*, BELLING and BLAKESLEE (9) and SATINA and BLAKESLEE (31) for *Datura* have reported that triploid plants of these genera show chromosome distribution at meiosis similar to that here found for *L. tigrinum*.

In later stages of development in the male gametes in triploids a wide variation in chromosome numbers has been reported. SATINA and BLAKESLEE on *Datura*, DARLINGTON (15) on *Hyacinthus*, LEVAN (20) on *Allium*, CAPINPIN (10) on *Oenothera*, and NAGAO (23) on *Narcissus* reported nuclei with all chromosomes, ranging from  $n$  to  $2n$ . In most of the investigations nuclei with lower chromosome numbers were found to be more numerous than those at the other limit of distribution. Not in all instances were the numbers of nuclei at or near the limits of distribution greater than expected.

The distribution of chromosomes at the metaphases of both the third and fourth nuclear divisions is similar in micropylar and chalazal nuclei, except that the effects of lagging and fragmentation are trebled in the chalazal nuclei, and as a result the shift to the lower chromosome numbers is more marked than in the micropylar nuclei (figs. 68, 69). Lagging in the nuclear divisions following meiosis has not been reported in previous investigations. In *L. tigrinum* such lagging occurs rather infrequently as compared with that in the meiotic divisions, and is possibly related to the chromosome unbalance and the existence of dicentric chromosomes and fragments. Actual counts were not made of chromosomes in the female gametes, but numbers at the metaphase of the fourth nuclear division should represent those counts rather accurately, since numbers would be modified so slightly in the fourth division.

Differential assortment of chromosomes of the type favoring the production of haploid and diploid gametes, as it occurs in *L. tigrinum*, may be explained if it is found that certain homologous chromosomes tend to pass together to the same pole. Behavior of this type has been reported in *Drosophila* by STURTEVANT (40) and in *Rosa* by HURST (19), but neither of these cases is comparable with that of *L. tigrinum*. In *Rosa*, chromosomes which failed to pair went together to the same pole. As shown in table 1, there is almost complete trivalency in the triploid *L. tigrinum*, indicating that the three sets of chromosomes making up the somatic number are very similar. There is, nevertheless, evidence that one set of 12 chromosomes may differ from the other two in regard to the structure of some of its members. Failure of correspondence in all chromomeres of a set of three homologous chromosomes has been mentioned. In previous investigations SATO (33) and CHANDLER, PORTERFIELD, and STOUT (11) also reported such apparent lack of strict homology in the prophase of the microsporocyte. They gave no definite suggestion as to the cause of the irregularity.

In the present investigation at early diakinesis of the megasporocyte three chromosomes were observed associated as shown in figure 66u, and as interpreted in figure 66v. Such configuration may result from a cross-over in an inverted segment distal to the centromere in the first of the three chromosomes. The break in the chromosome is supposed to have occurred at the point *x*, and an apparent lack of symmetry at this point—which corresponds in position to the end of the second chromosome—gives additional evidence in support of this supposition. The occurrence of chromatid bridges and fragments at the anaphase and telophase of both the first and second meiotic divisions adds confirmatory evidence for the existence of an inverted segment in at least one of the chromosomes.

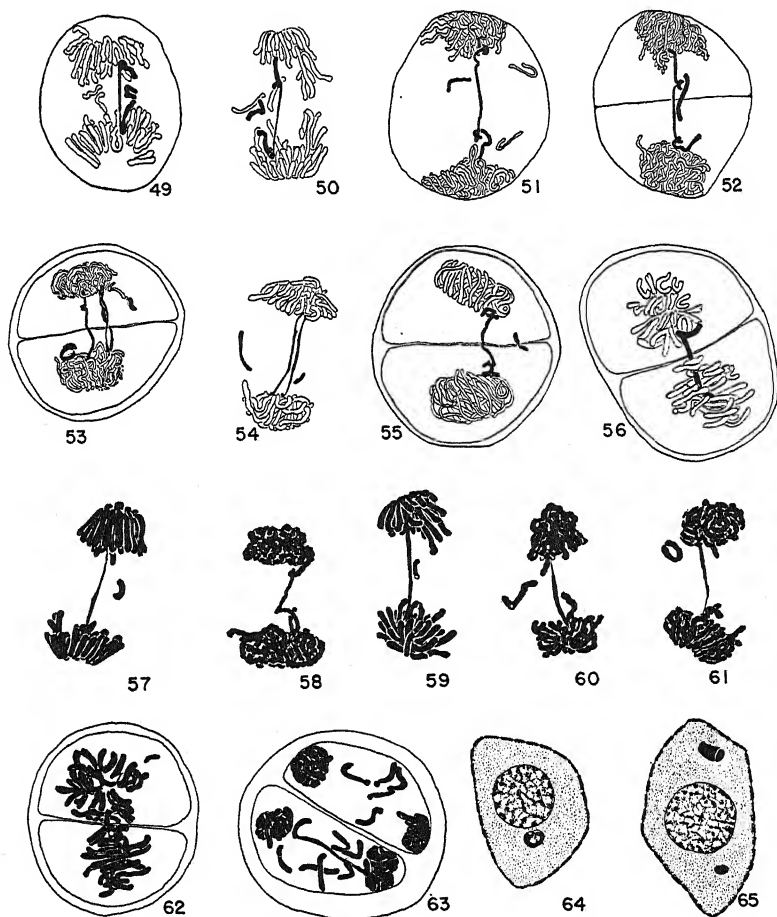
The relationship of bridging and fragmentation to crossing-over in an inverted segment distal to the centromere of one of the chromosomes is shown in figure 66, columns I, II, III, and IV. In the first three columns only two of the three homologous chromosomes are considered. In column I is diagrammed the possible sequence of events after the formation of one chiasma at pachytene (*c*), followed by bridge formation and fragmentation in metaphase and anaphase (*d*, *c*). In column II a similar sequence is shown following formation

of two chiasmata. The final configuration is the same in both cases. The acentric fragment may vary in length, depending on the distance from the end of the chromosome at which the chiasma in the inverted portion forms. Nuclei showing bridges and acentric fragments which may have been formed according to the plan of columns I and II are shown in figures 21, 22, 23, 30, and 49-60.

In column III of figure 66 is shown a complementary cross-over, resulting in the formation of two bridges and two acentric fragments. Cases of double bridge formation are shown in figures 53 and 54, and in *D* and *E* of figure 67. In column IV of figure 66 and in figure 67 are presented types of separation possible when three homologous chromosomes are associated as shown in figure 66*u*. It is probable that the two chromosomes joined by a chromatid connection tend to move together to the same pole in the first meiotic division, in which case the bridge is not evident until the second meiotic division. It may then occur in one of the two dividing nuclei (figs. 26, 27, 63).

If the two centromeres of the bridge-forming chromosome should always pass to opposite poles in the second meiotic division (fig. 67*J*, *K*), the ratio of bridges in the first and second meiotic divisions would form an index measuring the degree of differential or random assortment at the first division. But if the two centromeres should pass to the same pole, as in figure 67*L*, which is probably more likely, then the number of bridges appearing stretched from pole to pole in the second meiotic division would represent only a portion of the dicentric chromosomes actually present. In order to compute the degree of differential or random assortment in the first meiotic division, the percentage of bridges apparent in the second meiotic division should be multiplied by the ratio of dicentric chromosomes present to those apparent as bridges. For example, following random assortment of the two centromeres of the dicentric chromosome at the second meiotic division, the ratio of dicentric chromosomes present to those apparent as bridges would be 2 to 1. If, as is likely, the assortment is differential in favor of the two centromeres passing to the same pole, the ratio would be more than 2 to 1. Counts of chromosomes in the microsporocytes show an average of 17 per cent bridging in the second division as compared with 11.6 for the

first. If the percentage of bridges apparent in the second division is multiplied by two to allow for random assortment, the ratio of



FIGS. 49-65.—Figs. 49, 50, first meiotic division of microsporocytes, showing lagging and bridging; figs. 51, 52, 55, bridging and acentric fragments in microsporocytes; figs. 53, 54, double bridging; figs. 56, 62, bridges persisting at metaphase of second meiotic division; figs. 57-61, late anaphase and telophase of first meiotic division, showing bridging and acentric fragments; fig. 63, lagging chromosomes and bridging in second meiotic division; figs. 64, 65, microspores showing chromatic masses in cytoplasm.

bridges in the first meiotic division is 11.6 to  $2 \times 17$ , or 11.6 to 34. This means that two chromosomes attached by a dicentric chroma-

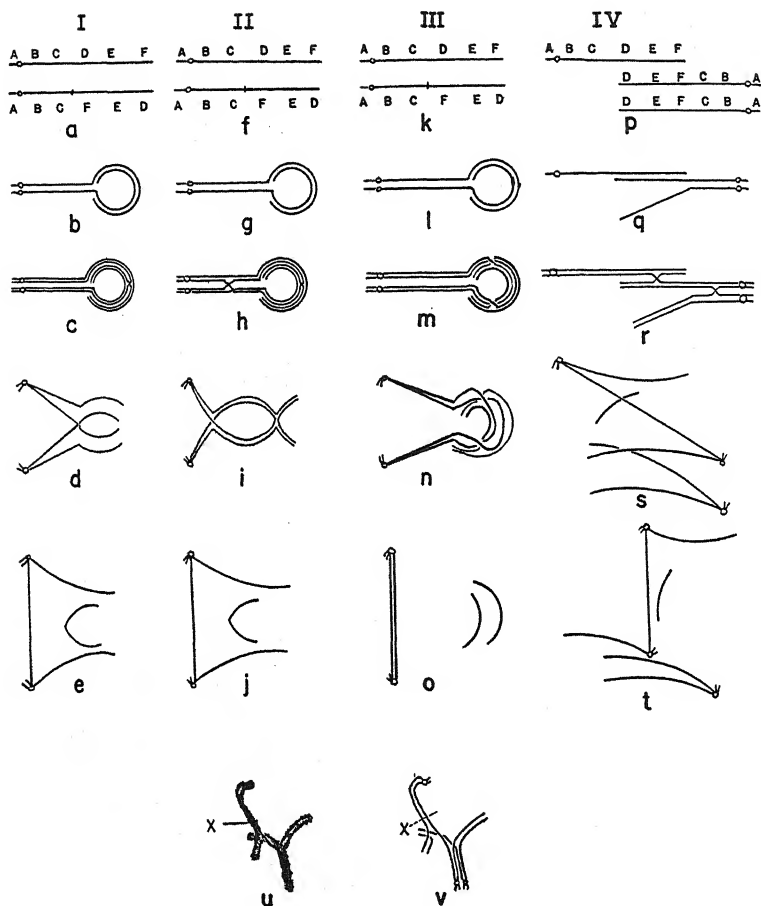


FIG. 66.—Diagrammatic representation of bridging as result of cross-over in inverted segment. Column I: *a*, two chromosomes showing reversal in segment of lower of the two; *b*, zygotene; *c*, pachytene with one chiasma in inverted portion; *d*, *e*, chromatid bridge and acentric fragment. Column II, sequence following formation of two chiasmata. Column III, double bridging following complementary cross-over. Column IV, sequence of events expected accompanying separation of trivalent chromosomes, one of which possesses inverted segment; *u*, observed trivalent association diagrammed in *v*, on which column IV is based.

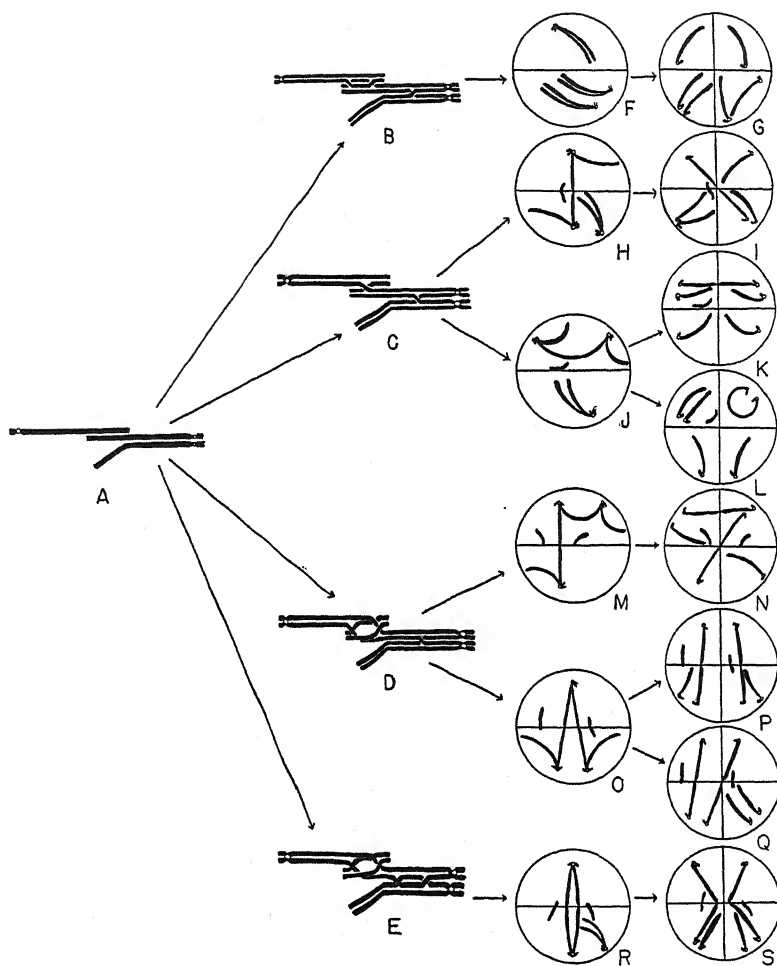


FIG. 67.—Possible meiotic distribution of three homologous chromosomes, one of which shows inverted segment: *A*, three homologous chromosomes arranged as in fig. 66, column IV; *B, F, G*, reciprocal crossing-over in which no bridges are formed; *C, H, I*, bridging at first meiotic division persisting until after the second; *C, J, K*, bridging at second meiotic division; *C, J, L*, no bridge formation following passage of the two centromeres of dicentric chromosome to same pole at both first and second meiotic divisions; *D, M, N, O, P, Q*, some possible arrangements of chromosomes following double bridge formation joining three chromosomes; *E, R, S*, double bridging resulting from complementary cross-over involving only two homologous chromosomes.

tid at the first meiotic division pass together to the same pole approximately three times more often than to opposite poles. If there is a similar differential assortment of centromeres of the dicentric chromosomes at the second meiotic division, only those whose centromeres pass to opposite poles are apparent as bridges, and the

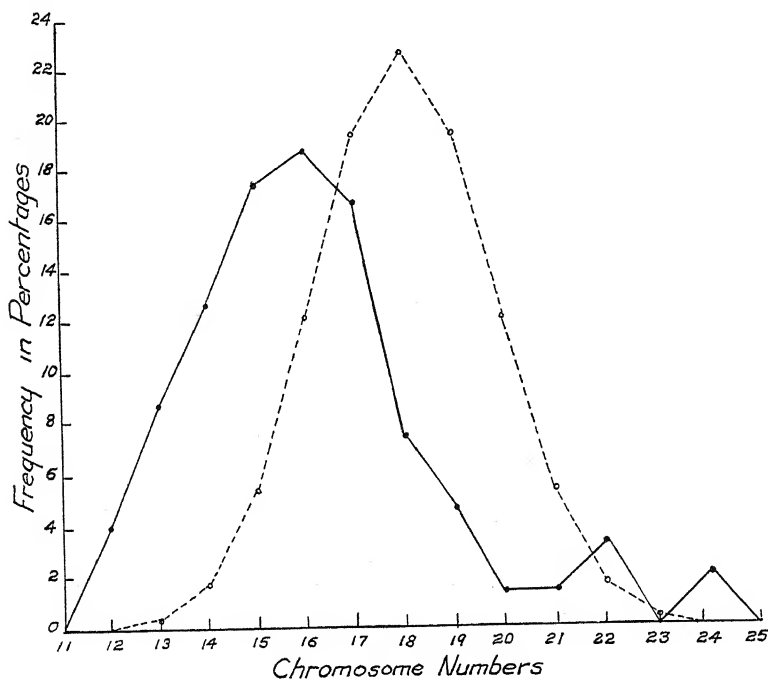


FIG. 68.—Graph showing distribution of chromosome numbers in 150 nuclei at micropylar end of megagametophyte, at metaphase of fourth nuclear division. Broken lines indicate distribution calculated from random assortment; solid lines represent observed frequencies.

index of differential assortment at the first meiotic division is considerably more than 3 to 1.

It is also possible that infrequently three homologous chromosomes become connected by two chromatid bridges (fig. 67*D*, *M*, *O*). Possible results of random assortment of the centromeres of the bridge-forming chromatids are shown in figure 67*N*, *P*, *Q*. More often the centromeres may all pass to the same pole, since all are

connected by bridges, and this would result in a decided differential assortment favoring formation of haploid and diploid nuclei and decreasing the number near the mean expected from random assortment. The nuclei with smaller chromosome numbers formed in this way would be non-functional as gametes, since they would not have a set of 12 different chromosomes. Half of the nuclei formed following the formation of two bridges (fig. 67*R, Q, S*) would also have an incomplete set of entire chromosomes. Although the oc-

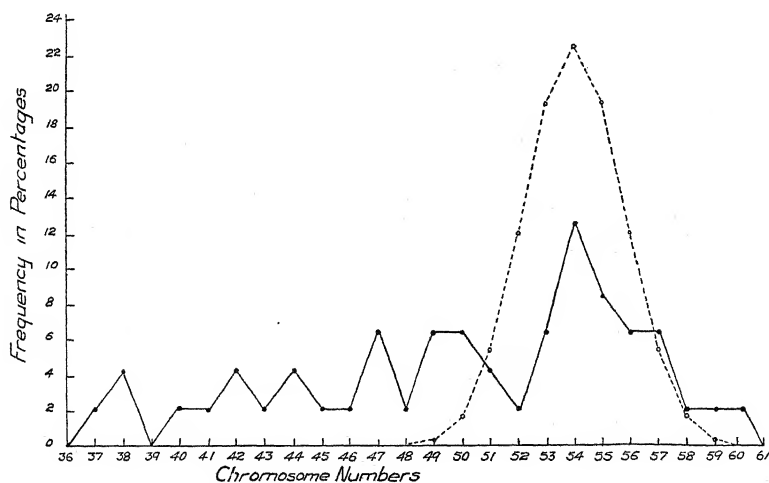


FIG. 69.—Graph showing distribution of chromosome numbers in 47 chalazal nuclei of megagametophyte, at metaphase of third nuclear division. Broken lines indicate distribution calculated from random assortment, solid lines represent observed frequencies.

currence of three homologous chromosomes joined by chromatid bridges may result in a more marked differential assortment in the particular cell in which it occurs than does the occurrence of two chromosomes so joined, the latter condition is so much more frequent that it probably influences differential assortment as a whole more. The numbers of haploid and diploid nuclei resulting from differential assortment would be approximately equal were it not for lagging and fragmentation, which decrease the number of nuclei with high chromosome counts and increase the number with low counts. It is therefore probable that the number of diploid progeny

of a cross between *L. tigrinum* and a diploid form would exceed that expected. Selective growth favoring haploid gametes at fertilization would also tend to produce diploid progeny. Evidence that the proportion of diploid plants resulting from triploid  $\times$  diploid crosses exceeds that expected from random assortment has been presented by BELLING and BLAKESLEE (9) in their work with *Datura* and by VAN OVEREEM (43) with *Oenothera*.

The presence of an inverted segment in at least one of its 36 chromosomes places *L. tigrinum* var. *splendens* in the structural hybrid class, notwithstanding the high degree of trivalency at diakinesis. Evidence for structural changes in chromosomes has previously been reported for several species of *Lilium*. RICHARDSON (27) reported inversions and duplications in the hybrid *L. martagon*  $\times$  *L. hansonii*. Failure of pairing has been reported for other species by HALL and MATHER (18), BELLING (8), SANSOME and LACOUR (28), and SATO (33). It is possible that many of the recognized species of *Lilium*, including *L. tigrinum*, are of hybrid origin. The prevalence of structural hybridity, although not conclusive evidence for interspecific hybridity, is at least of suggestive significance.

Bridging and fragmentation have been reported in structural hybrids of other genera. Recently WOODS (44), in an investigation of the triploid tulip Inglescomb Yellow, reported both bridging and fragmentation. As is true of *L. tigrinum*, the Inglescomb Yellow tulip showed a high degree of trivalency at diakinesis. In another recent investigation EMSWELLER and JONES (17) reported bridging in a hybrid between *Allium cepa* and *A. fistulosa*.

A close parallelism is evident between meiotic irregularities associated with structural hybridity due to inversion and irregularities commonly reported in triploids at meiosis. Among these irregularities are formation of univalents, which results in lagging chromosomes and subsequent production of micronuclei and microcytes. A second case of parallelism occurs in the type of departure from random assortment of chromosomes. The formation of bridges has also been reported in both types. These similarities in behavior suggest that structural hybridity may occur in triploids more often than has been supposed.

### Summary

1. Development of the megagametophyte in *Lilium tigrinum* var. *splendens* follows the general plan reported for the other members of the genus and for *Fritillaria* and *Tulipa*.

2. At metaphase of the first meiotic division 36 chromosomes generally occur in 12 trivalent groups, although bivalents and univalents may occur.

3. Distribution of chromosomes at the first meiotic division approaches random assortment, except that there are marked increases toward the  $1n$  and  $2n$  limits of distribution.

4. The chromatids of lagging chromosomes may separate precociously following the first meiotic division, after which they may pass tardily to the poles or remain in the cytoplasm between the two nuclei.

5. Lagging chromosomes and fragments eliminated from nuclei may disintegrate or persist as micronuclei.

6. Elimination of chromosomes through lagging and fragmentation in the meiotic divisions and to a less marked degree in the divisions following meiosis produces a general shift in the distribution toward the lower chromosome numbers.

7. Lagging chromosomes and bridging occur in the divisions following meiosis in the development of the megagametophyte.

8. The occurrence of chromatid bridges and fragmentation of chromosomes at meiosis results from crossing-over in an inverted segment of one of the chromosomes.

9. Crossing-over in an inversion results in the formation of dicentric chromosomes at meiosis in a triploid and thus favors the formation of haploid and diploid gametes.

10. There is close parallelism between meiotic irregularities found in structural hybrids and irregularities common to triploids.

The writer gratefully acknowledges the assistance and encouragement of Dr. J. M. BEAL during the course of this investigation.

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# STRUCTURE OF FOSSIL STEM OF PITHEAN AFFINITY FROM THE REED SPRINGS FORMATION OF MISSOURI<sup>1</sup>

J. E. CRIBBS

(WITH NINETEEN FIGURES)

## Introduction

The petrified stem described in this paper is from the Reed Springs Formation of the Mississippian Series. Three other stems have been described (4, 5, 6) from the same horizon. Two of those previously reported were found imbedded in chert, which forms more or less continuous seams in the limestone of this formation. This stem was found lying free among an accumulation of fragments which had broken up under the influence of weathering after the limestone had dissolved.

The specimen probably represents part of the main trunk of a small tree which was broken transversely by fractures subsequent to fossilization. The portion found (fig. 14) was about 35 cm. long, 9.5 cm. wide, and 6.5 cm. thick. Erosion has so affected the stem as to remove all structures external to the secondary wood, and an unknown amount of the latter has weathered from the surface, leaving a zone of unequal thickness. The stem is straight, approximately uniform in thickness, and without visible branches.

The petrification is siliceous, like that of other stems from chert seams of the Reed Springs Formation. There is a further resemblance in the bruised condition of the secondary wood, a feature which is particularly evident in sections. Apparently a certain amount of decay took place before infiltration, so that pits of both primary and secondary elements are poorly preserved, and are clearly visible in limited areas only.

Compression flattened the stem slightly, crushed part of the pith cells, produced four longitudinal fractures, and affected all of the

<sup>1</sup> This investigation was aided by a grant from the American Association for the Advancement of Science, awarded through the Missouri Academy of Science.

tissues in local areas. Spherulites occur throughout the specimen. About the margin of the pith, and especially in the primary bundles, there is an accumulation of dark materials which at places obscures the cell arrangement.

SECTIONS PREPARED.—In all, fifty-five sections were prepared, all cut on the petrotome. The peel method was not used because of partial decay of the organic material before petrification. After cutting the stem transversely, one of the two pieces was broken along a fracture which extended lengthwise near the center of the pith. The cut end surface of one piece was polished to show the general topography of the transverse sectional area. A large thin section was prepared (a composite) to show the number and arrangement of primary bundles. This included all the pith and the first growth zone of secondary wood surrounding it.

Twelve transverse sections were cut in series from a block 28 mm. long and 23 mm. wide, which included pith. These were prepared to determine the arrangement of primary bundles at the nodes, and to observe the general features of leaf trace emission. Because of certain irregularities that appeared in this series, it was supplemented by thirty-three tangential sections cut so as to show all the outgoing traces in a segment of stem 18 cm. long which included 5 cm., or about two-fifths of the pith circumference. Eighteen of these are thin sections cut between the pith margin and a point in the secondary wood about 4 mm. beyond the terminus of leaf trace bundles. Fifteen are polished surfaces of blocks cut about 2 mm. from the pith, and were prepared to determine the relative position of outgoing traces, whether the traces are single or double, and if possible to secure information regarding phyllotaxis. The remaining sections include tangential cuts of the pith and primary bundles; transverse sections of secondary wood; and radial sections of reparatory strands, outgoing traces, and secondary wood.

### Observations

#### PITH

Maximum dimensions of the pith as seen in transverse section are 50 mm. by 22 mm. (figs. 7, 15). In the central region is a band of crushed cells lying in the plane of greatest diameter, which apparent-

ly was parallel to the bedding plane of the stem before it was dissociated from the chert matrix. This band is about 5 mm. wide. Fine compression lines form a fan opposite each end of the crushed zone and extend outward to the secondary wood. Within this region the pith cells are somewhat distorted and structural features are partially obscure. Surrounding the central region of compressed cells, the pith varies from 5 mm. to 1 cm. in width and is composed of cells which are elongated radially, except for about 1 mm. adjoining the secondary wood where their greatest dimension is in the vertical axis and the radial elongation is less evident.

There are no sclerotic tissues or secretory ducts of any kind in the pith, which is perfectly continuous except for an irregular longitudinal fissure that developed where the fracture planes intersect near the center of the stem.

The boundary between pith and secondary wood, as seen in transverse sections, is not a smooth curve. It is characterized by shallow embayments five or six of which are visible in transverse section (fig. 7). These may be due in part to alteration during compression, but for the most part they represent embayments attending leaf trace emission. They affect the inner growth zone of secondary wood, beyond which the successive zones appear as smooth concentric lines since they are not traversed by outgoing leaf trace bundles.

The average dimensions of pith cells are as follows: tangential width 118.6  $\mu$ , radial width 264.6  $\mu$ , and length (height) 142.8  $\mu$ . Within 1 mm. of the margin the average dimensions are 84  $\mu$  tangential width, 142  $\mu$  radial width, and 168  $\mu$  height.

#### PRIMARY BUNDLES

In a transverse section of the stem ninety-one primary bundles are visible in the pith a short distance from the margin, and seven others are in contact with the secondary wood (fig. 7). The latter have not at that level given off a reparatory strand. Probably two or three additional bundles were present and were lost in the development of the fractures.

The bundles are submarginal in position and vary considerably in their distance from the pith margin. Most of them are 1 mm. or more from the wood and some are 2-3 mm. distant. They are dis-

tinctly isolated. There is not the tendency to become confluent as in the semi-herbaceous *Calamopityeae* (10, 13). The average distance of separation is about 1 mm., which is approximately five times their tangential diameter.

Where unaffected by compression, the primary strands are more or less circular in outline as seen in transverse section. Some of them are elliptical, with the greater dimension in the radial plane (fig. 12).

The bundles are typically mesarch with the protoxylem approximately central. There is some variation, however, so that occasionally they approach the endarch arrangement. The endarch tendency occurs only in the region where they curve out from the pith to the secondary wood, and where they divide tangentially to form leaf trace and reparatory strand (fig. 5).

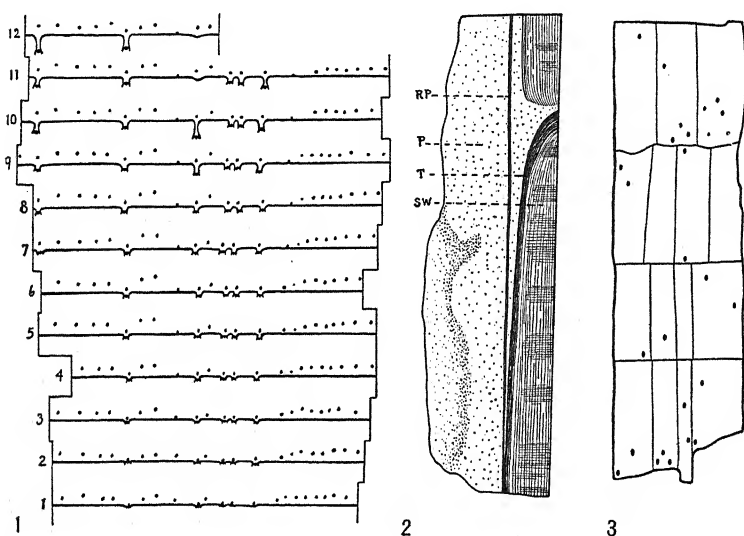
Those bundles which lie 1 mm. or more from the wood are within the zone where pith cells are radially elongated, and cells adjoining the strand commonly radiate from it as a focal point, or they may not be so elongated on the tangential borders. Nearer to the pith margin, cells adjacent to the bundles are not conspicuously elongated but tend to conform to the neighboring arrangement, being slightly longer in the vertical axis and approximately round in transverse section.

In the series of twelve transverse sections cut from a block 28 mm. long and 23 mm. wide (fig. 1), a maximum of twenty-three primary bundles appear in section 9. In this portion of the stem the bundles all follow a vertical course and there are no indications of lateral connections. A submarginal bundle turns out to the pith margin a considerable distance below the node (probably about 3 cm.), as seen in figure 4, and coincident with the formation of an embayment in the wood it divides tangentially to form an outer leaf trace and an inner reparatory strand (figs. 2, 6). The reparatory strand continues vertically, lying directly opposite the trace, and gradually comes to lie deeper in the pith. Each emerging trace shows the same structural arrangement.

In the composite section of the entire pith cylinder (fig. 7) there are five double bundles which lie at considerable distance from the secondary wood. Therefore branching of the submarginal bundles is known to have occurred sparingly at other places than the nodes.

The primary bundles are slightly larger in the upper part of their course, averaging  $190\ \mu$  (fig. 5), but the enlargement does not attain the dimensions described for the various *Calamopityeae*. As shown in figures 8 and 11, in the outgoing trace, where largest ( $400\text{--}600\ \mu$ ), they resemble those of *Callixylon* (1).

The size, large number, and submarginal position of the bundles agree with the condition originally described for *Pitys antiqua*. There

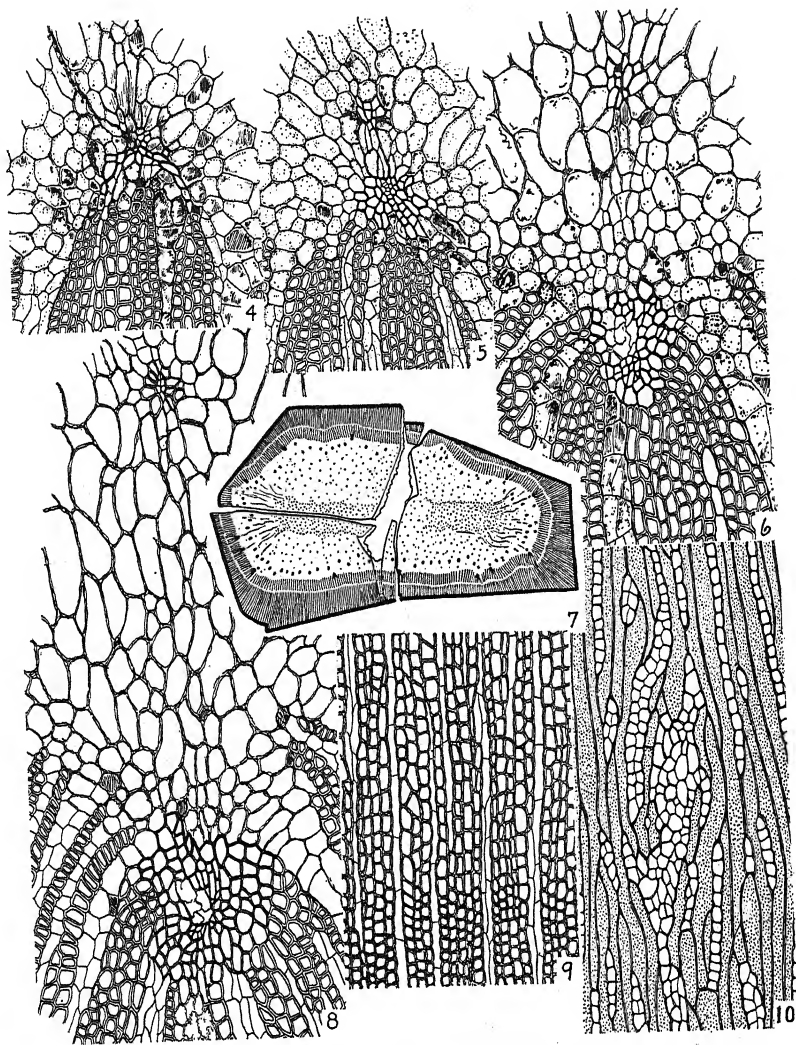


FIGS. 1-3.—Fig. 1, reconstruction of twelve transverse sections from a block  $28 \times 23$  mm., showing arrangement of primary bundles in relation to outgoing traces. Note vertical course and lack of interconnections. Fig. 2, reconstruction of radial section through reparatory strand, trace, pith, and secondary wood (*rp*, reparatory strand; *t*, trace; *p*, pith; *sw*, secondary wood). Fig. 3, reconstruction of fifteen blocks, representing fragment of stem  $5 \times 18$  cm. at pith border; traces shown as black elliptical dots; note paired arrangement except for two clusters.

are, however, no deeply imbedded medullary bundles such as have been described in *Archaeopitys eastmanii* (11, 12) and more recently in all the known species of *Pitys* in which primary tissues are preserved.

#### LEAF TRACES

A marginal bundle divides tangentially to form an outer leaf trace and an inner reparatory strand (fig. 5). The trace bundle, which is the larger, begins at once to sink into the secondary wood. It forms



FIGS. 4-10.—Fig. 4, transection of primary bundle (mesarch) where it turns out from pith to inner border of secondary wood, below point of division into reparatory strand and trace;  $\times 53$ . Fig. 5, formation of reparatory strand and trace; latter is the larger and lies in contact with secondary wood;  $\times 53$ . Fig. 6, beginning of trace emergence; trace somewhat enlarged with a few centrally placed parenchyma cells; eccentrically mesarch trace;  $\times 53$ . Fig. 7, composite showing transverse section of entire pith, first growth zone of secondary wood, and primary bundle arrangement;  $\times \frac{1}{2}$ . Fig. 8, later stage of trace emission showing embayment in secondary wood, mesarch trace, and reparatory strand deep in pith;  $\times 53$ . Fig. 9, transection of secondary wood showing border between summer and spring wood;  $\times 50$ . Fig. 10, tangential section cut about 2 mm. from terminus of leaf trace, showing slight distortion of secondary elements;  $\times 53$ .

an acute angle with the stem axis, and does not reach the outer edge of the first growth zone of secondary wood (3 mm. thick) until it has passed through a vertical distance of 2-2.5 cm. As a trace approaches the outer margin of the first growth zone, it curves sharply and assumes a position almost but not quite horizontal.

A small group of parenchyma cells is included with the primary xylem of the trace (figs. 6, 7). The mesarch arrangement typical of the primary bundles is continued in the trace, where the protoxylem lies immediately outside the parenchyma, and a second protoxylem center appears to be present in some instances, when it lies on the centripetal side of the parenchyma cells. There is a strong tendency for the centrifugal metaxylem to develop in radial alignment with the secondary elements, a feature characteristic of certain Cordaiteae (9).

As the trace emerges, a fan of secondary wood is carried out on the abaxial side, but so far as can be determined does not develop adaxially. At the outer border of the wood, an outgoing trace with its secondary elements has an average diameter of about 600  $\mu$ . Some of them do not exceed 400  $\mu$ . There is very little distortion of tracheids or rays attending the emergence of traces. On the abaxial side of the trace, where such distortion is commonly greatest, the rays are slightly wider and somewhat shorter than elsewhere; opposite their terminus in the wood secondary elements close over the break quickly, so that the tracheids appear quite normal about 2 mm. external to that point (fig. 10). Since the traces terminate at the outer border of the first growth zone, it is probable that the leaves were shed at the close of a growth season and were replaced by others at a higher level when growth was resumed.

There is a peculiar grouping of certain traces as seen in the series of transverse sections (fig. 1), where they lack the regularity normal to a spiral arrangement. Six traces pass out in the block, all emerging at about the same level. Their spacing in relation to the primary bundles is also irregular. Between the two traces shown at the left of the figure there are four intervening bundles; between the second and third there are three bundles; between the third and fourth there is one bundle; while the fourth, fifth, and sixth are derived from successive strands. Two bundles, one on each side of the

clustered traces, are in contact with the wood and presumably emerged slightly higher in the stem. None of the seven shown at the right have approached the wood preliminary to emerging. One bundle, the seventh from the right of section 2, diminishes in size upward and apparently terminates blindly, as it consists of a single tracheid in the last section and holds to a vertical course. Since its origin is not shown in this series, there is some doubt, but it probably is derived from a neighboring strand by division such as is seen to occur in the composite section previously referred to. If this is the true interpretation, it may represent in a much reduced form the condition described for *Archaeopitys eastmanii* (11, 12).

Because of the peculiar grouping of traces in this portion of stem, additional information was sought by cutting into suitable blocks a considerable portion of the stem obtained by breaking it along the main longitudinal fracture. These blocks, fifteen in number, were then so cut on the tangential plane as to show the traces on their way through the first growth zone of secondary wood. Figure 3 shows the relative position of the blocks and represents about two-fifths of the pith circumference. The traces, as shown in the figure, mark the relative levels at which they passed from the wood into the cortex, but their horizontal spacing corresponds to their distance apart at the pith margin where they connect with reparatory strands.

With the exception of two groups, including nine traces above and five below, they appear to be paired in arrangement, one lying at a higher level than the other. This arrangement resembles that described for *Pycnoxylon* (5), except that in this stem the traces are separated by one to four intervening bundles. It is possible that on their way through the cortex the traces changed their relative position, so that each entered a separate petiole. This appears doubtful, and the grouping can best be explained as a paired arrangement.

In regard to the two groups of traces there is considerable obscurity. They may represent bundles that passed out into a cauline appendage which may have been a fruiting stalk such as occurs in the Cordaiteae (9). The close grouping cannot be explained as due to excessive local compression that affected their relative positions, for the five shown at the upper right in the figure were in a region

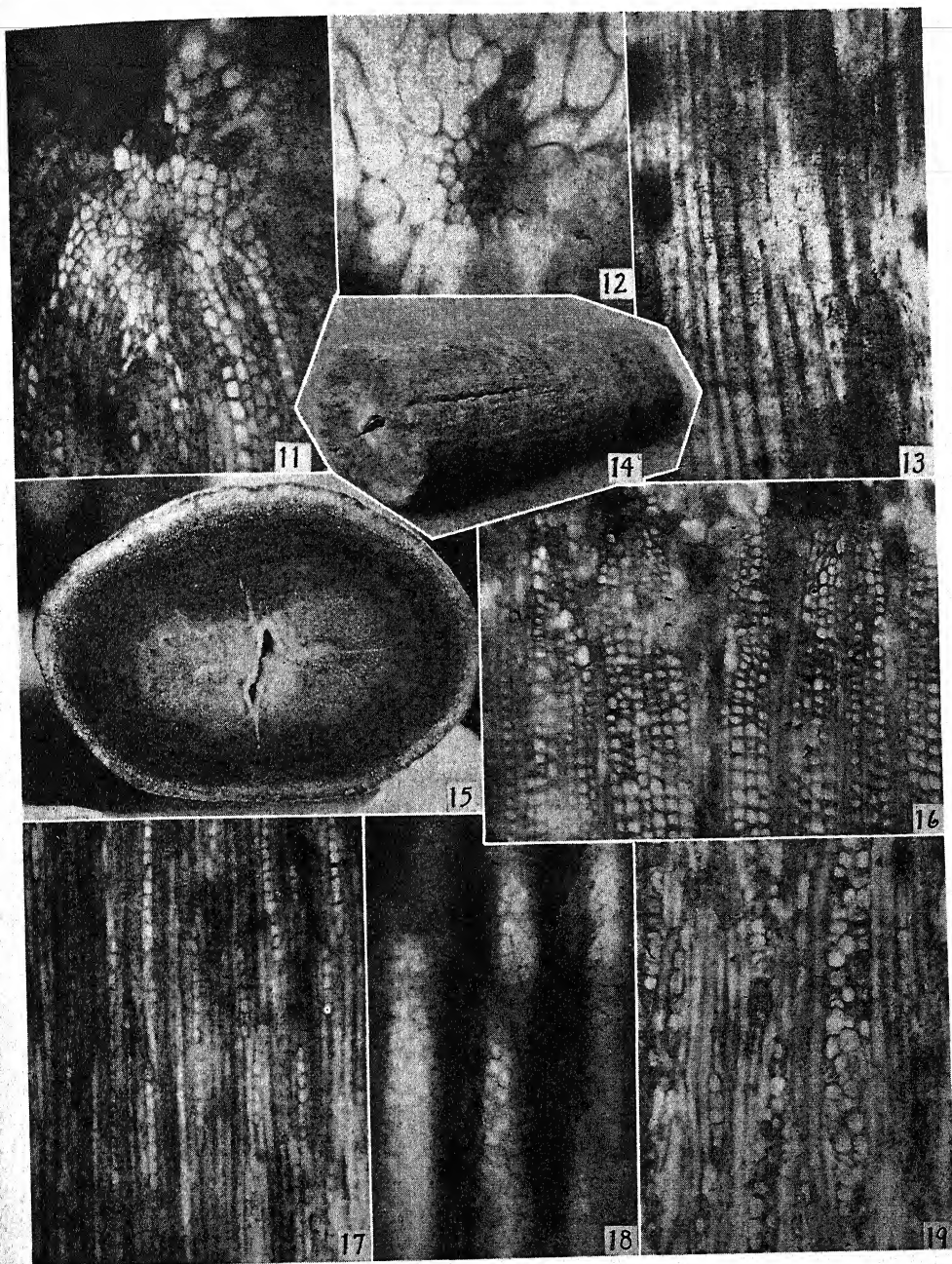
not affected by compression, and the group seen in the transverse series are shown to be derived in part from successive primary bundles. If they passed out into a fruiting stalk, that structure must have been herbaceous and caducous, for the bundles terminate in the secondary wood at the outer border of the first growth zone in the same manner as the others and there is no evidence of the secondary wood being involved, except for a small amount which is incorporated in the trace bundle. They are closed over by the second growth zone exactly like the traces. It is not probable that these grouped traces connected with buds, for in that instance they would doubtless bear a more consistent relation to the leaf traces, and if they extended into lateral vegetative branches there would be an extensive involvement of secondary wood. That these strands all supplied leaves is scarcely tenable, for obviously it would imply local irregularity in spiral arrangement. To the writer the most plausible explanation is that regarding a fruiting appendage.

The leaf arrangement is obscure, but apparently it is to be interpreted as a complex spiral, with paired traces one more apical than the other, and each of the pair given off from a separate primary bundle.

#### SECONDARY WOOD

The zone of secondary wood has a maximum radial thickness of 2.8 cm. and a minimum of 1.5 cm., an unknown amount having been lost previous or subsequent to fossilization.

Six concentric zones are visible in sections (fig. 15), and extend in a uniform manner about the stem. They are growth zones caused by the alternation of wider (spring wood) and narrower (summer wood) elements. As indicated in figure 9, the transition from summer to spring wood is definite but not always abrupt. The larger tracheids include about eight to ten cells in radial series. Their average radial dimension ( $60\ \mu$ ) is about twice that of the smaller tracheids. There is a gradual transition from larger to smaller elements. The zonation appears more distinctly under a hand lens than when observed under higher magnification. This is due to the absence of inclusions in the larger tracheids, whereas those formed later are commonly filled with dark materials which may be interpreted as stored substances or as organic matter derived from the thick walls, which broke down in part during the process of infiltration. The



FIGS. 11-19.—Fig. 11, transection of emerging trace showing mesarch arrangement and a few parenchyma cells included in bundle, also tendency for centrifugal metaxylem to be radially grouped;  $\times 52$ . Fig. 12, mesarch bundle in submarginal position; outline is elliptical with longest dimension in radial plane;  $\times 56$ . Fig. 13, radial section of secondary wood with crowded multiseriate pits uniformly distributed;  $\times 59$ . Fig. 14, general view of stem as found;  $\times 1$ . Fig. 15, transverse cut of stem showing large pith and growth zones;  $\times \frac{1}{4}$ . Fig. 16, transection of secondary wood at pith border showing slight dilation of rays which are at most three cells wide;  $\times 52$ . Fig. 17, tangential section of secondary wood about 1 cm. from pith, showing rays narrow and high;  $\times 44$ . Fig. 18, radial view of bordered pits with slitlike pores;  $\times 235$ . Fig. 19, tangential section of secondary wood and rays cut close to pith border; ray cells approximately isodiametric;  $\times 52$ .

zonation is less sharply defined than in modern conifers, but is more definite than in most Paleozoic woods thus far described. The first zone is about 3 mm. wide, the second 7 mm., the third 3 mm., and subsequent zones average approximately 2 mm. in width.

Neighboring wood rays, as seen in transverse section, are separated by four or five radial rows of tracheids which average  $29\ \mu$  in tangential and  $32.5\ \mu$  in radial diameter. They are roughly rectangular, with the corners rounded.

Wood rays are dilated slightly where they meet the pith (figs. 16, 19), owing in part to the ray cells being wider in this region, and also to the frequent occurrence of rays that are three cells in width. Within 1 mm. of the pith, the wider rays are narrow, commonly uniseriate, and their cells are radially elongated. Except near the pith margin, rays are typically uniseriate or biseriate in part, and often of great height. Rays thirty to fifty cells high are common and occasional ones exceed ninety cells in height (fig. 17). The ray cells where uniseriate average about  $40\ \mu$  high and  $30\ \mu$  wide, except near the pith where they are approximately  $70\ \mu$  in each dimension (fig. 19). Well out in the secondary wood where the ray cells are muriform, they have transverse or sloping end walls, and average about  $125\ \mu$  in radial length. No ray tracheids have been found.

On tracheids of the secondary wood, pits are typically limited to the radial walls, are uniformly distributed, crowded and hexagonal, or occasionally rounded and contiguous (fig. 13). They are most frequently arranged in three vertical series and alternately grouped. The pore is an inclined slit (fig. 18). Tangential pits occur sparingly on tracheids abaxial to an outgoing trace. The protoxylem bears spiral markings, and the metaxylem of the submarginal bundles is mostly transitional between reticulate and pitted. True pits could not be found in the metaxylem except in the outgoing trace where they occur on both radial and tangential walls. The transition from spiral elements to pitted in the primary wood of the trace takes place much more quickly than is characteristic of *Cordaia* (9).

#### RELATIONSHIP

In many structural features this stem resembles *Pitya*, and more particularly *P. withami* (8) from the Lower Carboniferous of Scotland. It has in common with *Pitya* a large number of mesarch

primary bundles buried in the pith near its margin. These bundles are small, of fairly uniform size, and turn out to the secondary wood zone only where they are about to connect with outgoing leaf traces. Other structural resemblance to *Pitys* is found in the large continuous pith; the prominent development of secondary wood; the formation of growth rings; parenchyma elements included in the outgoing traces; and metaxylem which is pitted in the trace but prevailing scalariform or transitional between reticulate and pitted in the submarginal bundles.

There are, however, several structural features which differ from those of *Pitys* and required the establishment of a new genus. The most obvious of these are: restriction of primary bundles to a submarginal zone; compact wood with uniseriate rays; paired leaf trace arrangement; position of reparatory strands; and the cytology of the pith.

In all species of *Pitys* so far as known there are in addition to the submarginal ring of bundles many others which in transverse sections appear scattered throughout the pith column (7). The primary bundles of this stem are all submarginal in position, but they lie somewhat deeper in the pith than corresponding bundles of *Pitys*. Six to thirteen cells may intervene between them and the margin whereas in *Pitys* two to three cells commonly separate them from the secondary wood. Certain of the primary bundles are smaller and more deeply imbedded than the others. At two or three places in the stem radial division of bundles is observed. Such division may be interpreted as a simplified pityean arrangement. Other bundles divide in a diagonal or tangential direction, however, and they may all be instances of lateral connections between neighboring strands.

The different species of *Pitys* are determined largely by relative width of the wood rays. Throughout the genus they are conspicuous, being widest in *P. rotunda* (7) and narrowest in *P. withami* (7). In the latter they may be six cells in width although most of them are biseriate. In this stem both primary and secondary rays are uniseriate or biseriate in part. In this respect it has a density similar to that of *Cordaites*. There is, however, this interesting transitional feature—the presence of rays of great height.

A paired leaf trace arrangement has not been described for any of the Pityeae although it is a common feature of Paleozoic seed plants.

It is typical of the Lyginopterideae and the more herbaceous Calamopityeae, both of which bear certain structural resemblance to this specimen. Paired traces also occur in the Poroxyleae and Cordaiteae of the Cordaitales. In the several species of *Pitys* (5, 6, 7) and *Callixylon* (2), and in *Archaeopitys* (11, 12), the traces—so far as they have been observed—pass through the wood singly, and in *P. dayi* (7) are known to divide external to the secondary wood. The occurrence of paired traces in wood of this stem thus tends to set it apart from the genus *Pitys*.

The manner in which reparatory strands are formed is almost identical with the condition described for *Callixylon erianum* by ARNOLD (1), but differs considerably from *Pitys* in which there is no reparatory bundle directly opposite the trace, continuity being maintained through lateral connections with adjoining primary bundles (7).

In all species of *Pitys* so far described the pith appears to be composed of three types of cells (7), scattered secretory elements, storage cells, and thin-walled parenchyma. The latter for the most part surrounds the primary strands. The pith of this stem is composed almost entirely of thin-walled parenchyma which is radially elongated. None of the secretory type have been observed, and storage cells occur sparingly near the pith margin.

Certain features of this stem resemble *Callixylon* more closely than *Pitys*. As indicated in a preceding paragraph, this is true of the reparatory strands. The restriction of primary bundles to the sub-marginal zone may be considered intermediate between *Pitys* and *Callixylon*, for in the latter they are grouped in a ring about the pith margin, some imbedded and others in contact with secondary wood. The dense wood with narrow rays resembles that of *Callixylon whiteanum* (3). In contrast with *Callixylon*, tracheids are absent from wood rays and pith, there is uniform radial pitting, and the leaf traces are paired.

This stem is from the same horizon as *Pycnoxylon* (5) and bears certain resemblance to it, particularly in the dense wood, large pith, and a paired trace arrangement. *Pycnoxylon* differs in having primary bundles endarch below the nodes, tracheids in some of the rays, reparatory strands lateral to the leaf trace, traces which pass out

forming a wide angle with the stem axis, primary bundles more closely associated with secondary wood, and paired traces that originate from the same strand.

Recently the writer has described another stem from the Reed Springs Formation (6). It is similar in certain respects to this specimen but because of features which indicate affinity with pteridosperm stock should be taken into consideration. It has in common with this stem a large pith (52 mm. diameter), one hundred or more submarginal bundles, and paired traces.

It further resembles *Pitys* in having wide rays, primary strands with lateral connections, and traces that pass out through the wood forming a wide angle with the stem axis. Affinity with certain Calamopityeae is indicated in the reduction of primary bundles below the nodes, an endarch tendency, sclerotic clusters in the pith, paired traces, and the evidence that leaves were large. Certain of these features may be interpreted as indicating either relationship or parallel development; for example, sclerotic clusters occur in the pith of *Eristophyton* and *Lyginopteris* but have not been reported as occurring in the pith of any of the Pityeae. Since they are present in the cortex of *P. dayi*, they may readily have been more extensively distributed in other members of the family. In similar manner the reduced state of the primary bundles with the attendant irregularity of protoxylem position may be interpreted as an adaptive variation associated with numerous bundles and very short internodes.

The occasional groups of traces which characterize this specimen probably connected with a fruiting branch of cauline origin. The manner in which reproductive structures were borne has not been determined for *Pitys* or *Callixylon*. In *Poroxylon* (9) adaxial buds which may have formed a fruiting branch have vascular connections with the two neighboring bundles, one in the anodic and the other in the cathodic position in relation to the traces.

Although the stem described in this paper shows certain affinity with pteridosperm stock, the very large pith, many primary strands, absence of pitted elements in the metaxylem of the bundles, and the arborescent tendency all indicate a closer affinity with the Pityeae than with any known members of the Lyginopterideae or Calamopityeae, with which there is a similarity in general organization.

Until a more extensive comparative study is completed on the Reed Springs flora, it seems advisable to place the stem tentatively in the Pityeae as a new genus and species for which the name *Megalomyelon myriodesmon* is proposed. The generic name refers to the large continuous pith, and the specific name to the very large number of small submarginal primary bundles.

***Megalomyelon myriodesmon* gen. et sp. nov.**

Pith 50 mm. wide, continuous, without ducts or sclerotic nests, cells elongated radially. Primary bundles, about 100 in number, mesarch, submarginal in position, becoming marginal only near a node, branch sparingly, not confluent, follow vertical course, protoxylem spiral, metaxylem transitional between reticulate and pitted. Reparatory strand single, smaller than leaf trace, mesarch, on same radial plane as outgoing trace. Leaf traces paired, one more apical than the other, derived from separate bundles, mesarch, not greatly enlarged at node, small amount of parenchyma included, metaxylem radially aligned centrifugally, slightly larger centripetally, pitted on all walls. Parenchyma embayment at pith margin continues as crescent over outgoing trace. Secondary wood of trace limited to abaxial side. Secondary wood abundant, with growth zones; spring wood average 60  $\mu$  radial diameter, summer wood 32.5  $\mu$ . Wood slightly distorted abaxial to outgoing trace, closes over trace terminus quickly with slight distortion. Wood rays at pith border one to three cells wide, approximately isodiametric, and prevailingly high (maximum ninety cells). Wood rays 1 cm. from pith uniseriate or biseriate in part, high (ninety cells maximum), muriform, about 125  $\mu$  radial dimension, transverse or sloping end walls, ray tracheids not observed. Pits prevailingly crowded, hexagonal, uniformly distributed, and commonly in three alternate series, occasionally rounded and contiguous. Pore an inclined slit, fully bordered. Leaf trace sequence interrupted by occasional clusters of traces.

**Summary**

1. The petrified stem the structure of which is here described came from the Reed Springs Formation of the Mississippian. It is infiltrated with siliceous materials and probably represents a portion of the main trunk. Since the cortical structures are lost and an unknown amount of the secondary wood has eroded, the maximum

diameter of 9.5 cm. indicates that the specimen represents a plant which originally had the dimensions of a small tree.

2. There is general agreement in most structural features with the genus *Pitys*, with which it is more closely related than with any other known form. The most significant points wherein it differs from *Pitys*, namely, paired leaf traces and narrow rays, are characteristic of *Cordaitea* on the one hand and of certain *Calamopityeae* on the other. The leaf trace arrangement is so unlike any known member of the genus *Pitys* that the stem is given generic rank, but because of the prevalence of pityean features is included in that family.

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# DETERIORATION OF GRASSLAND FROM STABILITY TO DENUDATION WITH DECREASE IN SOIL MOISTURE<sup>1</sup>

J. E. WEAVER AND F. W. ALBERTSON

(WITH SEVENTEEN FIGURES)

## Introduction

The great drought has now prevailed intermittently in the mid-west for a period of seven years. Damage to native vegetation in 1934 has been partially repaired, but further destruction has also occurred. Continued study of the shiftings in dominance among the grasses has revealed widespread losses of certain species and partial or complete replacement by others. Forbs also have greatly diminished, both in number of species and in abundance, and changes in structure of vegetation generally have been pronounced (3, 4). Hence it seemed advisable during 1939 to study the degree of deterioration of the grasslands, if any, from western Iowa, through eastern Nebraska and Kansas, to western Kansas where in places almost complete destruction of vegetation has occurred. This study included monthly determinations of water content of soil to the depth of root penetration of the grasses, and other major environmental factors characterizing the growing season. The rate of growth of vegetation and density of cover were determined, as well as the distribution of forbs.

Groups of prairies whose previous history was known were selected for study (fig. 1). Soils were all similar, being of silt-loam texture; although those at Lincoln, Nebraska, and eastward belong to the Prairie zonal group, while those under lighter precipitation westward are Chernozems. All are of high fertility, and water is the chief limiting factor to plant growth.

<sup>1</sup> Contribution no. 121 from the Department of Botany, University of Nebraska. This investigation was aided by a grant to the senior author from the Penrose fund of The American Philosophical Society.

### Environmental relations

The critical environmental factors characteristic of the drought are low unevenly distributed precipitation, deficiency of soil moisture and accompanying high temperatures, high evaporation rates, and increased wind movement.

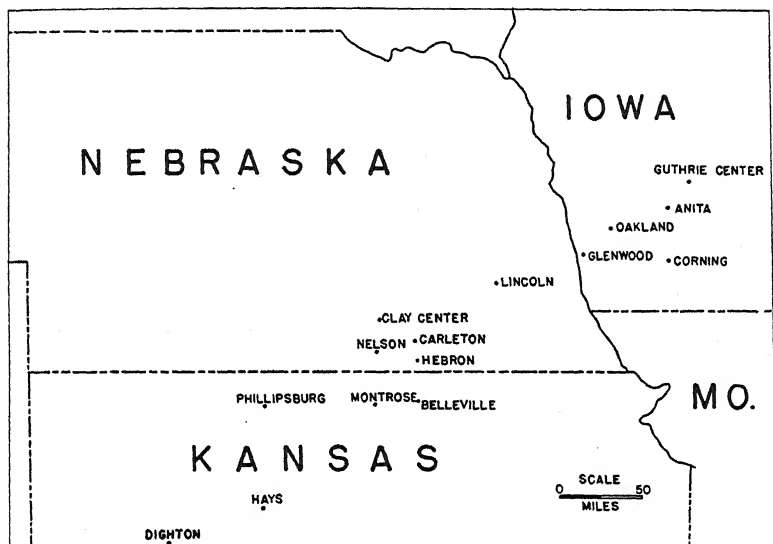


FIG. 1.—Map showing the four groups of stations where studies were made in 1939. Iowa group and Lincoln-Hebron-Nelson-Belleville group were dominated by *Andropogon scoparius* and *A. furcatus*. *Agropyron smithii* was dominant at Clay Center-Carleton-Montrose group, and *Bouteloua gracilis* and *Buchloe dactyloides* at the three most southwesterly stations.

### PRECIPITATION AND WATER CONTENT OF SOIL

Stations for water-content sampling were selected with reference to typical and relatively uniform composition of vegetation. In each group of stations were included various gentle slopes and nearly level uplands, where water content of soil depended entirely upon the precipitation in that area. Samples were secured regularly near the middle or latter part of each month from April to August, inclusive.

At the five stations in Iowa, where the mean annual precipitation ranges from 29.5 to 33.7 inches, rainfall during the several months

was sufficient to keep both soil and subsoil constantly moist to a depth of 6 feet at least (fig. 2). In April, at all stations the surface 24 inches of soil had 10-20 per cent or more moisture available for growth, and the deeper soil 2-10 per cent or more.<sup>2</sup> During the entire growing season, soil moisture depleted by the growing vegetation was restored more or less regularly and completely. To a depth of 24 inches, the available supply ranged continuously between 2

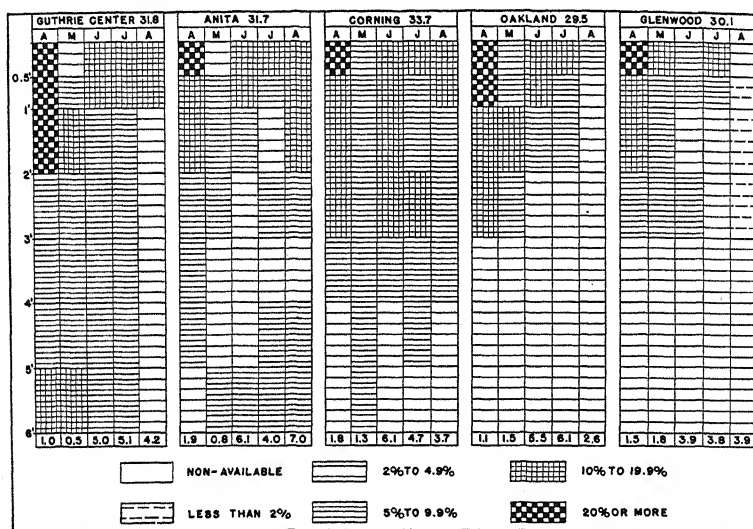


FIG. 2.—Available soil moisture to depth of 6 feet at the five Iowa stations during 1939. Mean annual precipitation at each station follows the name, and total current monthly rainfall is given at the foot of each column.

(but usually 5) and 10 per cent or more. A single exception occurred at the most westerly station (Glenwood), where in August water content was reduced to a minimum of less than 2 per cent. Water in the deeper soil was also always available, usually in the amount of 2-5 per cent or more. This moderate water content of soil resulted not only from the rather regular and well distributed rainfall, but also from its ready entrance into these silt-loam soils, which were kept in a porous, water-receptive condition by the continuous cover of grass.

<sup>2</sup> Total water content of soil minus the hygroscopic coefficient (which was determined for each soil depth at each station) is designated as water available for growth. The hygroscopic coefficients, with few exceptions, ranged between 9.1 and 12.9 per cent.

The mean annual rainfall at the second group of stations varies from 26.2 to 27.9 inches. Precipitation is sufficient, if well distributed, to keep the soil continuously moist under a cover of grassland, but this was a season of drought. Rainfall of June alone was equal to (or slightly below) the normal at all stations in this group (fig. 3). Early spring sampling showed an available water supply of 2-20 per cent or more in the surface 24 inches, except at the Kansas station

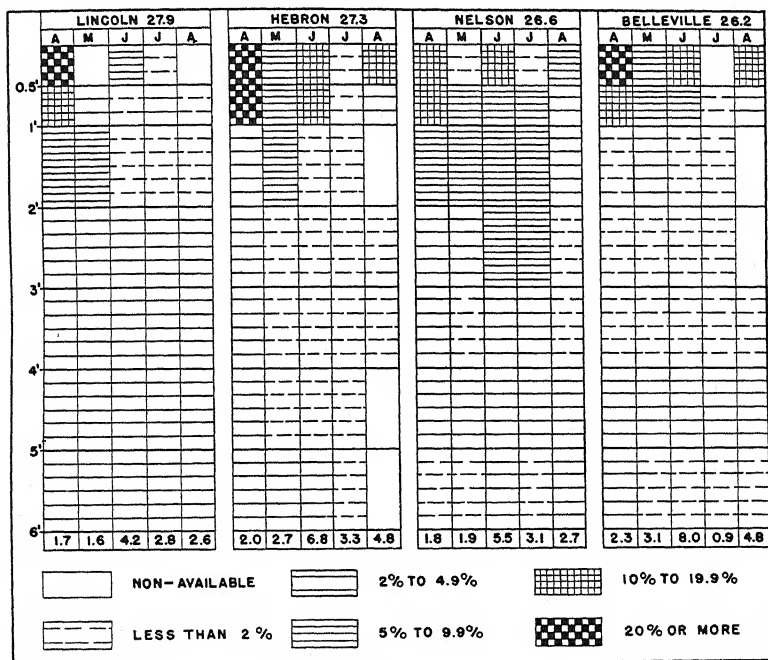


FIG. 3.—Available water content of soil and mean annual and current monthly precipitation at Nebraska-Kansas bluestem stations in 1939.

(Belleville) where the second 12-inch level had less than 2 per cent. The subsoil to 6 feet had consistently 2 per cent or more of available water, except at three levels at two stations where it was less than 2 per cent (fig. 3). The surface 24 inches maintained a good water content continuously at only one station (Nelson); at the others it was reduced to the point of nonavailability at some level one or more times, and at several samplings to less than 2 per cent. In the subsoil at Lincoln a small supply (2-4.9 per cent) was continuously available to 6 feet; at the other stations available water was fre-

quently reduced to less than 2 per cent, and at some periods none was available at certain levels.

While reduction in water content was brought about by a considerably depleted plant cover, much bare ground was also present. This decreased the efficiency of absorption of water to a considerable degree and promoted greater loss by runoff. Very numerous experiments, at practically all of the stations in the second and third groups, showed that within the same square meter water penetrated one and a half to two or even three times as rapidly where grasses still clothed the soil as where the ground was bare.

The three stations, Clay Center, Carleton, and Montrose, form a natural grouping, despite the fact that the mean annual precipitation at Carleton is slightly above that of two in the preceding group (fig. 4). The dominant vegetation at these stations is western wheat grass (*Agropyron smithii*) and not bluestems (*Andropogon scoparius* and *A. furcatus*), as at all the preceding prairies. Hence an important factor affecting the early exhaustion of soil moisture was, unlike that of bluestems, the very early and rapid growth of the wheat grass.

A good available water content occurred in the surface foot in April (10 to more than 20 per cent). Available water was sufficiently replenished at Carleton so that at least very small amounts continuously occurred in the first foot, but at the other stations complete exhaustion over long periods was repeatedly found. At 1-2 feet in depth, small amounts of water were available except in August at Carleton, but moisture was never available at this level at Montrose. Below 2 feet, no water was available for growth at any level of sampling after April, except less than 2 per cent at 5-6 feet depth at Carleton and similar small amounts (or none) at Clay Center.

These prairies had not only suffered greater losses of plant cover by previous drought than the preceding group, but also a rather complete change in plant population. This resulted in part from a cover of dust of variable thickness which clogged the soil pores so effectively that much of the torrential rainfall never entered the soil but was lost as runoff. Moreover, it has been found that wheat grass furnishes only poor protection to soil from surface puddling

when water falls upon it. Compared with the rate of penetration in adjacent, small, relict areas of bluestems, the period required for the entrance of similar amounts of water (as in light to moderate showers) is one and a half to two times as great.

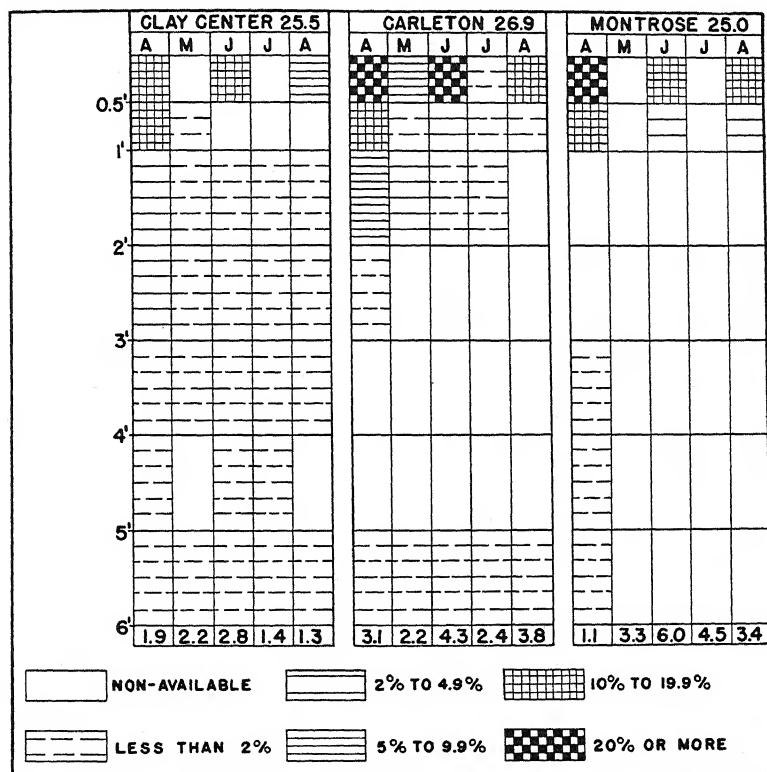


FIG. 4.—Mean annual precipitation, current monthly precipitation, and available soil moisture at Nebraska-Kansas wheat-grass stations in 1939.

The last group of stations has a decidedly lower mean annual precipitation, 18.9–23.3 inches, than any of the preceding (fig. 5). The mixed prairie grassland has here been more or less completely reduced, either by the recent drought (Phillipsburg) or by long continued grazing and drought (Hays and Dighton), to a short-grass plains disclimax. On each prairie enough silt has been deposited as dust to seal the soil so effectively that runoff has been greatly in-

creased. It was further accelerated by the small amount of vegetative cover and consequent lack of much obstruction to water movement. Thus, despite the rather high rainfall of June, the surface soil alone was moistened. Usually in this more arid region much of the precipitation is of low efficiency, because it falls either as torrential showers with high runoff or in many small showers followed by

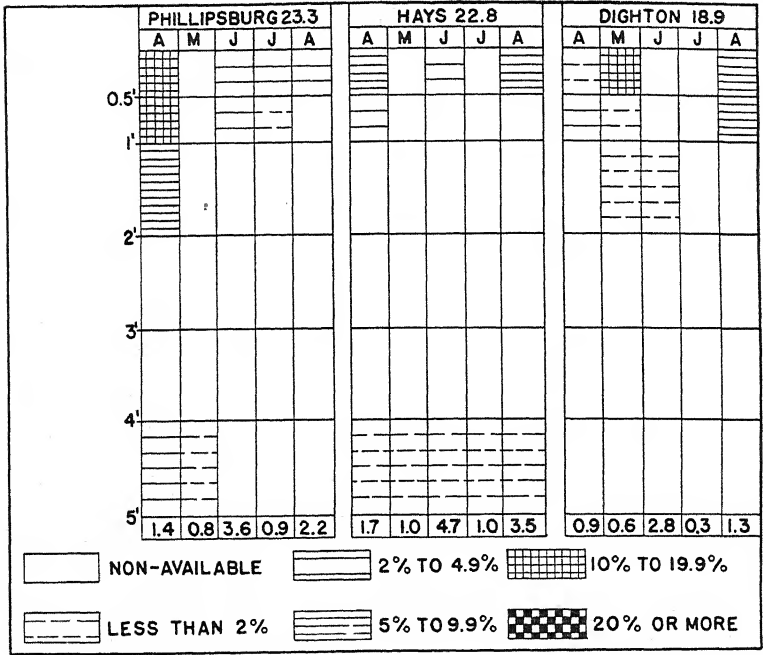


FIG. 5.—Chart showing relatively small amounts of water available for growth of vegetation in western Kansas and lack of subsoil moisture in 1939.

bright sunshine and often by high winds; hence the water is soon lost from the soil by evaporation. For example, the 4.7 inches of rain in June at Hays fell on thirteen different days. Only four of the rains exceeded 0.3 inch in amount and the heaviest for any day was 1.5 inches.

The surface foot of soil was moist (mostly from melting snow) in April, but only slightly so at the most westerly (Dighton) station. At various times throughout the summer no surface soil moisture was available for growth. The deeper soil, with rare exception, had

no available water. There was no reserve, even in early spring. At Hays it seems that a small amount of residual moisture remained unused at the 5-foot depth.

The charts present an accurate general picture of water content of soil. Weekly determinations add but little information to the general sequence. At Hays, for example, weekly samplings showed that during the first three days of May small amounts of water were available in the surface 6 inches. Water was also available the first five days of July and unavailable the last four days in August. At the second 6-inch level it was available in small amounts in the first week of May. Otherwise the weekly data conform entirely with the chart.

Thus while the grasslands of Iowa had an abundant to moderate but continuous supply of available soil water, those in the second group had—after early spring—only a low supply and were repeatedly threatened with drought. At the third lot of stations, available subsoil moisture was the exception; at Carleton the surface foot was usually moist, but complete exhaustion occurred repeatedly in the other prairies. Water was available only in the upper soil at the most westerly stations and only at two or three periods during the growing season.

#### TEMPERATURE, EVAPORATION, AND WIND

Mean temperatures for April were practically normal, and only  $2.7^{\circ}$  F. (or less) higher in the most southerly group of stations ( $53.7^{\circ}$ ) than in the eastern or two central ones. They varied less than  $1^{\circ}$  among groups during May, but at all stations they were about  $5^{\circ}$ – $8^{\circ}$  higher ( $68^{\circ}$  F.) than normal. A difference of only  $2.5^{\circ}$  between the lowest and highest average temperatures occurred in June, the highest average ( $75.2^{\circ}$ ) being recorded in west-central Kansas. At all stations, temperatures were only  $2^{\circ}$ – $4^{\circ}$  above normal. During July, temperatures from Iowa to western Kansas ranged from  $77.9^{\circ}$  through  $82.8^{\circ}$  to  $84.0^{\circ}$ . They were approximately  $3^{\circ}$ – $6^{\circ}$  higher than normal, the increase being slightly the greatest in western Kansas. August was cooler, mean average temperatures being  $72.0^{\circ}$ ,  $75.6^{\circ}$ , and  $77.9^{\circ}$  in the preceding sequence. Variation at any station above or below the normal did not exceed  $2^{\circ}$ .

Maximum temperatures during periods of stress were neither so

high nor of so great duration as during 1934 or 1936. The writers believe that high temperatures in grasslands have not been the direct cause of death of plants, but that they are merely one of several factors which intensified drought. Death due directly to drought resulted from lack of water. Where a water supply was artificially maintained for native species, no injury has been found.

Water loss as measured by rate of evaporation was much greater at the westerly stations than at those eastward. During April and May it was 39-51 per cent higher at Hays than at Lincoln. In June and July it was about twice as great at Hays, the difference in rate decreasing to 63 per cent in August. The lowest loss at Hays was 8.9 inches from a free water surface in April; the highest 20.6 inches in July.<sup>3</sup>

Comparison of the average daily losses from May to August inclusive during the extremely dry, hot summer of 1934 and that of 1939 is pertinent. These were measured by white, spherical, porous cup atmometers. Evaporation in 1934 was 82 cc., in 1939 only 49 cc. During the hottest month of 1939 (July) the average daily loss was 48 cc., compared with 96 cc. in 1934. Compared with evaporation losses during 1933 (just preceding the severest year of drought), weekly averages were higher only three times. During the period of greatest stress (third week of July), however, losses were 88 cc., twice as great as during the same period in 1933.

Wind movement increases westward. During April, 1939, it was about twice as great at Hays as at Lincoln. In May and June it was about three times as great, in July three and a half times, and in August there was more than three times as much wind movement westward. From April to August inclusive it totaled over 28,000 miles at Hays, at Lincoln about 10,000.<sup>3</sup> Wind is increasingly detrimental to vegetation westward, not only because of the damage done by wind-carried dust but also because of frequent high velocities immediately following showers. This increases both evaporation and transpiration, which in turn accelerate the depletion of soil moisture.

While neither wind movement nor evaporation losses were as great as during certain other drought years, at most stations at

<sup>3</sup> Data from U.S. Department of Agriculture, Weather Bureau.

least they were considerably greater than normal during the pre-drought period. Loss of vegetative cover increased wind movement near the soil and greatly accelerated direct loss of soil moisture by evaporation. Had the soil provided ample water for absorption, however, it seems certain that vegetation would have suffered little, even under these conditions of somewhat increased temperatures, greater stress of evaporation, and increased wind movement.

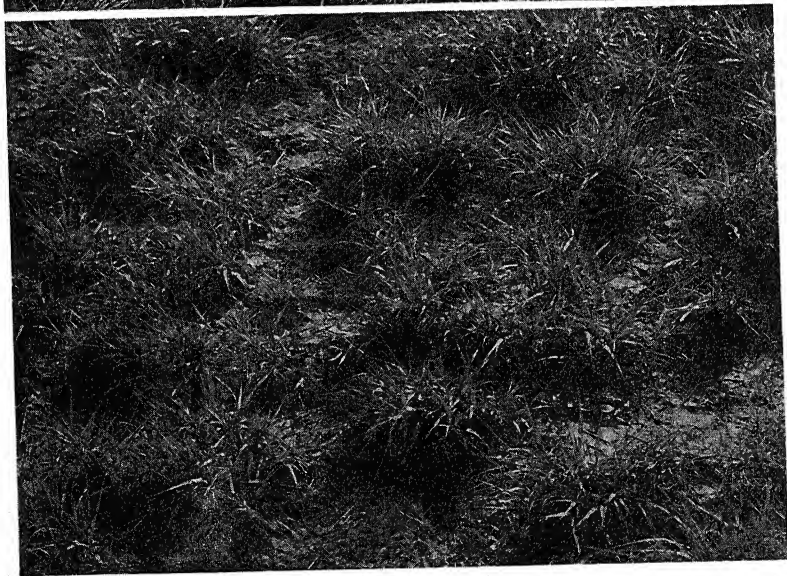
#### Previous deterioration of plant cover

The Iowa prairies had not been affected as regards change of structure by the great drought of 1934 or subsequently. An exception was some death on the drier slopes at Corning, but complete recovery had occurred by 1939 (3). In spring, following the preceding mowing in fall, the soil at all the stations was completely obscured by stem bases and a continuous cover of fallen debris a few millimeters in thickness. There was no bare ground (figs. 6, 7). Numerous lichens and mosses were found on the damp spongy earth between the tufts of sod. By midsummer the tall dense cover of vegetation, with a wonderfully developed understory and an abundance of forbs overtopping the grasses, revealed the prairies as of old (5). At no time during the entire growing season was plant development retarded by drought.

Deterioration of the prairies of Nebraska during the drought of 1934 and the results of the drought in both Nebraska and Kansas during the following year have been described (6, 3). With successive dry years further deterioration had occurred (4). At the second group of stations in the spring of 1939, much soil was bare. Soil-sampling stations were selected in the more stable portions of each of the four prairies where the cover of bluestems, although greatly thinned, remained intact. A chief change was an increase of other grasses, especially *Bouteloua curtipendula*. Widely spaced bunches and tufts of grasses with much bare soil, almost without protecting debris, were the characteristic features (figs. 8, 9). The basal cover had been reduced to two-thirds to one-half its former area. The ground layer of low-growing species and of lichens and mosses in spring had practically vanished. The remaining vegetation produced much less debris. The lack of moisture and shade caused it to



FIGS. 6, 7.—Fig. 6 (above), basal cover at Guthrie Center, Iowa, late in April, 1939. The bluestems, etc., were mowed late in fall of preceding year, yet the leafy stem bases and fallen debris completely conceal the soil. Fig. 7 (below), similar view at Oakland, Iowa, April 21. Note complete cover of dormant grasses and leaf mulch.



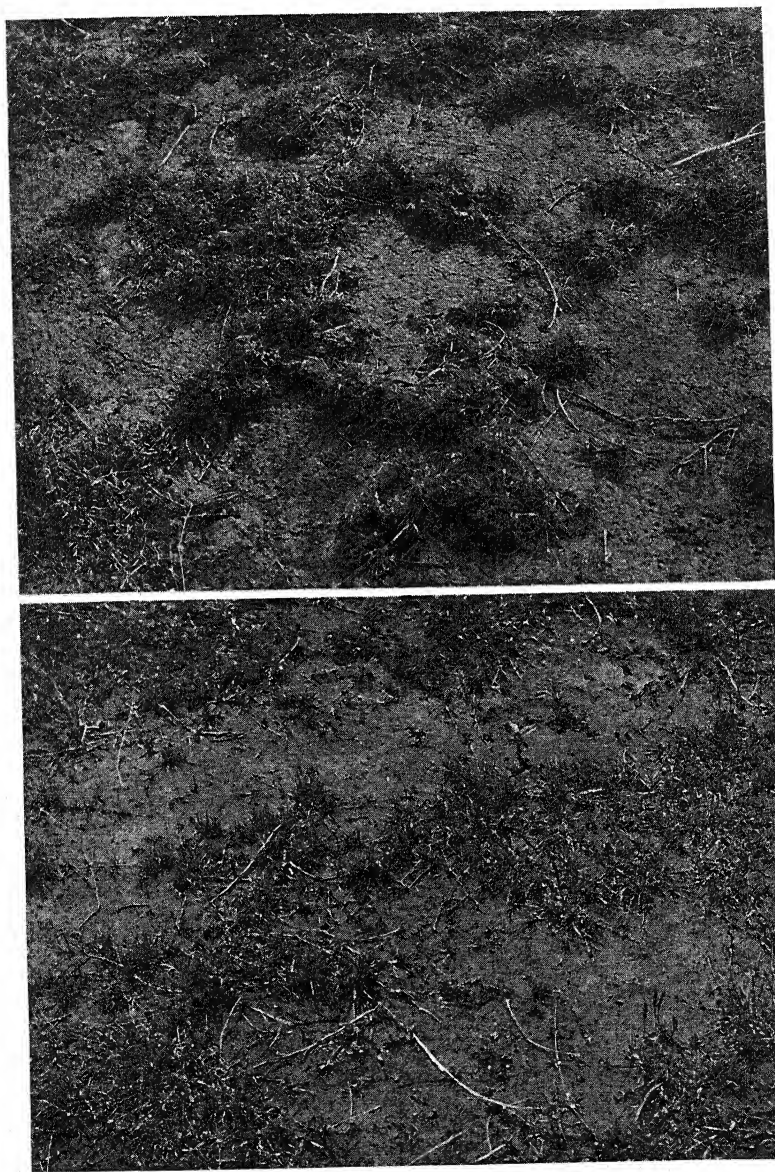
FIGS. 8, 9.—Fig. 8 (above), basal cover of little bluestem and other grasses which had made some fall growth after early mowing in autumn of 1938. Soil between bunches of grass is almost bare. Photographed Lincoln, Nebraska, April 29, 1939. Fig. 9 (below), similar view at Nelson on June 16, 1939. Widely spaced bunches and bared soil permit much loss of precipitation by runoff.

shriveled and dry, and high winds carried it away, leaving the ground quite bare. Each successive year of drought had further reduced a once abundant growth of forbs, until the grasses almost alone constituted the mid-layer, which was overtopped sparingly by small remnants of the once abundant taller forbs.

Great modifications, involving an almost complete change in the species of dominant grasses, had occurred at the three more xeric stations—Clay Center, Carleton, and Montrose. The bluestems had been all but exterminated by drought and deposits of dust, the Carleton prairie being least affected by dust burial. A wave of *Agropyron smithii* had swept over these prairies, taking rather complete possession, but this grass was being partly replaced by rapid spread of *Bouteloua curtipendula* (2). Sufficient moisture had not been available for the sod-forming wheat grass to aggregate densely. Between the widely spaced stems much unoccupied soil occurred, other plants had nearly all died, litter was scarce, and bare ground formed a continuous pattern everywhere.

Drought and dust had caused greater deterioration at the western Kansas stations than in any other group. The ungrazed prairie at Phillipsburg had lost nearly all its mid grasses (except *B. curtipendula*) and was largely converted into an open stand of the short grasses—*B. gracilis* and *Buchloe dactyloides* (fig. 10). Subsequently side-oats grama (*Bouteloua curtipendula*) had increased greatly. Only a single layer of vegetation remained, however, since these grasses alternated and did not intermix, while forbs occurred only sparingly. Where *B. curtipendula* grew, only 10 per cent of the soil was occupied, the wide spacing being an adaptation to drought. The patches of short grasses were so widely spaced that they probably occupied only about 10 per cent of the total of the remaining surface, since many irregular areas of a few square feet to a few square yards were entirely bare.

The two short grasses in the moderately grazed range at Hays were formerly intermixed almost equally to form a basal cover of approximately 85 per cent (1). This had been reduced by a light cover of dust and drought to only 16 per cent (fig. 11). In 1939, blue grama (*Bouteloua gracilis*) formed three-fourths of the vegetation, buffalo grass (*Buchloe dactyloides*) having suffered the greater losses.



FIGS. 10, 11.—Views of short grasses showing conditions of vegetation at most westerly group of stations early in June, 1939. Fig. 10 (above), nearly pure *Bouteloua gracilis* in lightly grazed range representative of this type at Phillipsburg, Kansas. Fig. 11 (below), *Buchloe dactyloides* in well kept pasture, illustrative of depletion of cover near Hays, Kansas.

Little bluestem (*Andropogon scoparius*), other mid grasses, and most of the forbs had succumbed to drought.

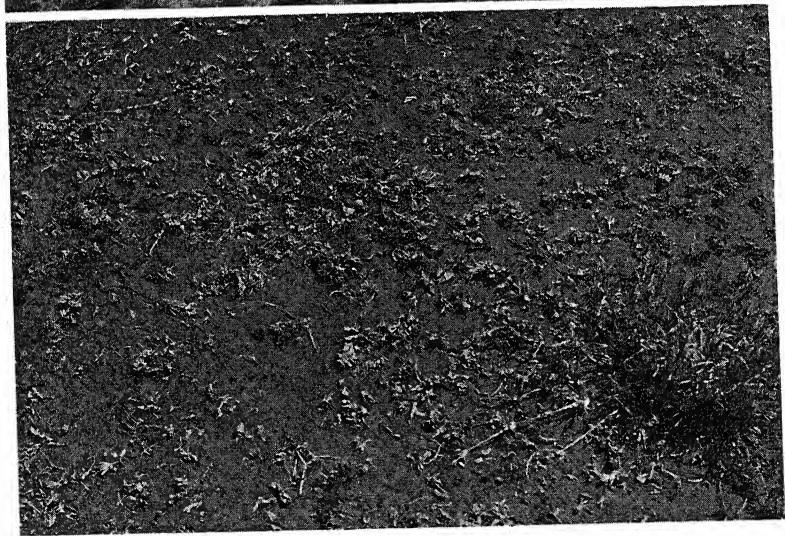
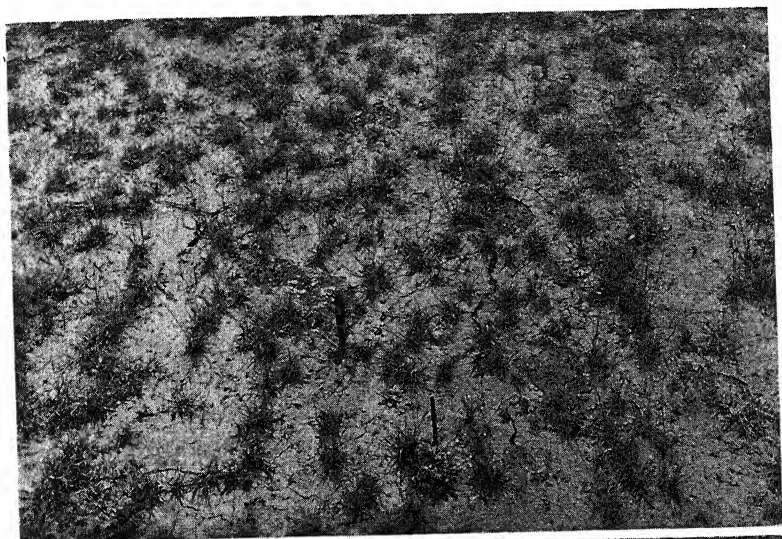
Blue grama and buffalo grass, in approximately the proportions of 3:1, constituted practically the entire cover of 12-15 per cent at Dighton. This prairie was also grazed, as are practically all the upland midwestern grasslands, since the forage is usually too short to cut for hay. At all three stations, plants of the lower layer had all but disappeared, and any debris from the scanty vegetation was swept away by high winds, leaving the surface nearly bare (figs. 12, 13).

Thus the preceding years of deficient moisture, great dust storms, and other unfavorable environmental conditions, while scarcely extending to the prairies of Iowa, had an increasingly deleterious effect toward the central area of greatest damage in western Kansas.

#### Plant development in relation to drought

Growth in spring was delayed at the Iowa stations because of late heavy snow. For most species it began late in April and progressed steadily, uninterrupted by drought at any time during the summer. A sprinkling of 2-5 per cent of *Poa pratensis* throughout and its blossoming at a height of 10-12 inches in May were evidence of the mesic conditions that prevailed. Height growth of the foliage of bluestem grasses was complete late in July, when a level of 15-20 inches was attained; after this flower stalks began to develop. The yield of forage was about normal, one to one and a half tons per acre.

Early spring growth was about normal at the second group of bluestem stations. The prairie vegetation was much less luxuriant because of the severe thinning of the grasses and the fact that the bunches were poorly filled with stems, both effects of the preceding drought. In Iowa, for example, a given basal area of little bluestem was 75-90 per cent occupied with leafy stems, but in this group the space was usually only one-third to one-half filled. Moreover the understory was represented by mere fragments or none of the usually widely distributed *Viola pedatifida*, *Antennaria campestris*, *Astragalus crassicaulus*, *Sisyrinchium angustifolium*, and the rosettes of *Hieracium longipilum*, *Erigeron ramosus*, and others. Precipita-



FIGS. 12, 13.—Fig. 12 (above), typical ground cover at Dighton, Kansas, August, 1939, on lightly grazed range. *Bouteloua gracilis* and *Buchloe dactyloides* constituted nearly all the scanty vegetation, but *Malvastrum coccineum* and a few other xeric forbs occurred. Eighty-eight per cent of soil is bare. Fig. 13 (below), almost complete loss of vegetation by burial with dust, which wind has now swept away, leaving dead bases of short grasses as evidence of formerly good cover. Photographed Sharon Springs, Kansas, June 2, 1939.

tion in May was deficient. Grasses of early growth, especially *Stipa spartea* and *Agropyron smithii*, soon exhausted their water supply, and late in the month were wilted and the leaves much discolored. *Stipa* produced very little seed, and flower stalks of *Koeleria cristata* were short. Abundant rainfall in June permitted normal development of the vegetation. Drought prevailed at several periods in July, with temporary revival of growth following showers. By mid-July both andropogons were wilting at most stations and many leaves were dead and brown. Pronounced drought injury was evident during August. The grasses dried more or less completely, after producing the reddish or brownish colors of late autumn, although some new greening of basal leaves occurred following showers in September. Flower stalks were not produced by the bluestems, except at Nelson, which had the best water supply (fig. 3). The formerly pronounced seasonal aspects were poor in spring and early summer, and there were none thereafter. Yield of forage was light, only one-third to one-half ton per acre.

Growth at the third group of stations (Clay Center—Carleton—Montrose) began about a month earlier than at the preceding one, since the controlling species is the early starting *Agropyron smithii*. Its dominance followed the death, by drought and (in part) burial by dust, of the bluestems. Loss of the bluestems was accompanied by death of a considerable portion of the population of forbs and other grasses. As a result of the lighter rainfall and the earlier absorption of soil moisture, growth was poor even in early summer. The spring aspect was poorly developed and lack of seasonal aspects characterized the remainder of the year. The wheat grass was 5 inches tall by April 12, at a time when the prairies were dry and the old foliage sprinkled with dust. Drought in May caused the leaves to roll tightly, and growth temporarily ceased. During June, when blossoming normally occurs, the foliage level was only 7–14 inches high and flower stalks were few or none, except at Montrose where a scanty crop was developed. During the remainder of the summer, the stems and leaves were dry and the intermixed side-oats grama—and even the blue grama—failed to produce flower stalks. No plant was in blossom; indeed the entire landscape presented no green color. An exception occurred at Carleton, where a good crop

of short flower stalks was produced by side-oats grama in July. At Montrose, a prairie fire burned clean everything above ground over half the area the first week in July. Of the few scattered forbs, all but those of spring failed to flower; grasses developed so poorly that the crop of hay was too scanty to warrant cutting.

Growth occurred only intermittently at the short-grass stations, since there was no reserve moisture and vegetation depended entirely upon the light current precipitation. A few vernal species with large storage organs, especially *Anemone caroliniana* and *Allium nuttallii*, grew in response to moisture furnished by melting snow, as did certain annuals, notably *Plantago spinulosa*, *P. purshii*, *Lepidium densiflorum*, and *Hordeum pusillum*. Few annuals, including *Salsola pestifer*, developed beyond the seedling stage or attained a height of more than 1-3 inches. Even the perennials with food reserves usually failed to produce flower stalks; only a few windflowers, for example, partly unfolded their blossoms on dwarfed stems which dried with the exhaustion of available water.

Rains of late May or early June promoted ephemeral growth of the dusty, dry short grasses, which was soon terminated by mid-summer drought. The prairies were sere and brown until once again the surface soil was moist in August. Then stolons of buffalo grass spread laterally 5-10 inches before the moisture was exhausted, the roots at the nodes becoming successively shorter and more subject to death from drying as the distance from the parent plant increased. Where some protection was afforded from too rapid drying, as about the bunches of cacti, blue grama grass put forth flower stalks and blossomed in late fall. But later examination revealed that few viable seeds were matured. The dwarfed, most xeric forbs grew precariously, with little or no blooming.

Thus, except for sporadic greening, these prairies were mostly in a condition of drought dormancy, with grass leaves rolled or folded. The short grasses, exclusive of the few flower stalks, were only 2-3 inches tall. In addition to light grazing by cattle (except at Phillipsburg), the leaf tips of the short grasses were often removed by grasshoppers, which occurred plentifully throughout the entire growing season, frequently at the rate of 6-15 per square foot. Little bluestem, wire grasses (*Aristida* spp.), and other mid grasses having

been killed by drought, the very open growth of short grasses alone partially covered the soil, except at Phillipsburg where short grasses alternated with side-oats grama. The impress of drought prevailed at all times. In fact, the much depleted cover, rather than being restored, apparently suffered further deterioration.

#### Grasses and forbs at the several stations

A census was made at each station, where soil samples were taken, of all species occurring within a radius of 100 feet. The lists of grasses and sedges and of forbs at the Iowa stations are shown in table 1. There are fifteen species in the first group. The forbs varied in number from thirty-seven to forty-eight at the individual stations and totaled sixty-five species. There were nineteen species of grasses at the Lincoln group of stations. Number of species of forbs varied from nine to nineteen and totaled thirty-three (table 2). In the group of wheat-grass prairies, the grasses and sedges were reduced to nine species. Species of forbs varied from eight to fifteen, with a total of twenty-one (table 3; figs. 14-17).

The forbs occurring at the three most westerly stations were few in number. Many less xeric species, common before the drought, were not found. The most evenly (although sparingly) distributed at the three sampling stations were *Malvastrum coccineum* and *Sideranthus spinulosus*. *Kuhnia glutinosa*, *Psoralea tenuiflora*, *Liatris punctata*, and *Cirsium undulatum* were found less abundantly, each at least at two of the three stations. *Allionia linearis*, *Allium nuttallii*, *Anemone caroliniana*, *Lygodesmia juncea*, and *Opuntia humifusa* occurred in the sampling area of at least one station. To this list of eleven perennial species must be added a half dozen (mostly native) annuals, previously mentioned, and the dominants *Bulbilis dactyloides* and *Bouteloua gracilis*; also at one station, *B. curtipendula*. Thus both reduction in number of species and sparseness of individuals toward the "dust bowl" were emphasized. Here, indeed, the web of life is stretched thin to cover the barren ground.

Increase in number of grasses from fifteen to nineteen from Iowa to Nebraska resulted from the gain of six xeric species (*Agropyron smithii*, two species of *Bouteloua*, *Buchloe dactyloides*, *Festuca octoflora*, and *Sporobolus asper*), but also included the loss of two (*Elymus*

TABLE 1

BLUESTEM PRAIRIES IN IOWA, SHOWING SPECIES OF GRASSES, SEDGES, AND FORBS AT EACH SAMPLING STATION: 1, GUTHRIE CENTER; 2, ANITA; 3, CORNING; 4, OAKLAND; AND 5, GLENWOOD

SPECIES	STATIONS					SPECIES	STATIONS				
	1	2	3	4	5		1	2	3	4	5
<i>Andropogon furcatus</i> .....	✓	✓	✓	✓	✓	<i>Euphorbia corollata</i> .....	✓	✓	✓	✓	✓
<i>A. scoparius</i> .....	✓	✓	✓	✓	✓	<i>Fragaria virginiana</i> .....	✓	✓	✓	✓	✓
<i>Bouteloua curtipendula</i> ...	✓	✓	✓	✓	✓	<i>Gentiana puberula</i> .....	✓	✓	✓	✓	✓
<i>Carex festucacea</i> .....	✓	✓	✓	✓	✓	<i>Helianthus rigidus</i> .....	✓	✓	✓	✓	✓
<i>C. pennsylvanica</i> .....	✓	✓	✓	✓	✓	<i>Heuchera hispida</i> .....	✓	✓	✓	✓	✓
<i>Elymus canadensis</i> .....	✓	✓	✓	✓	✓	<i>Hieracium longipilum</i> .....	✓	✓	✓	✓	✓
<i>Eragrostis spectabilis</i> .....	✓	✓	✓	✓	✓	<i>Houstonia angustifolia</i> .....	✓	✓	✓	✓	✓
<i>Koeleria cristata</i> .....	✓	✓	✓	✓	✓	<i>Kuhnia glutinosa</i> .....	✓	✓	✓	✓	✓
<i>Muhlenbergia cuspidata</i> .....	✓	✓	✓	✓	✓	<i>Lepachys columnaris</i> .....	✓	✓	✓	✓	✓
<i>Panicum scribnerianum</i> .....	✓	✓	✓	✓	✓	<i>Lespedeza capitata</i> .....	✓	✓	✓	✓	✓
<i>P. wilcoxianum</i> .....	✓	✓	✓	✓	✓	<i>Liatris pycnostachya</i> .....	✓	✓	✓	✓	✓
<i>Poa pratensis</i> .....	✓	✓	✓	✓	✓	<i>L. scariosa</i> .....	✓	✓	✓	✓	✓
<i>Sorghastrum nutans</i> .....	✓	✓	✓	✓	✓	<i>L. squarrosa</i> .....	✓	✓	✓	✓	✓
<i>Sporobolus heterolepis</i> .....	✓	✓	✓	✓	✓	<i>Linum sulcatum</i> .....	✓	✓	✓	✓	✓
<i>Stipa spartea</i> .....	✓	✓	✓	✓	✓	<i>Lithospermum linearifoli-</i> <i>um</i> .....	✓	✓	✓	✓	✓
<i>Acerates</i> spp. ....	✓	✓	✓	✓	✓	<i>Lygodesmia juncea</i> .....	✓	✓	✓	✓	✓
<i>Achillea occidentalis</i> .....	✓	✓	✓	✓	✓	<i>Meibomia canadensis</i> .....	✓	✓	✓	✓	✓
<i>Amorpha canescens</i> .....	✓	✓	✓	✓	✓	<i>M. illinoensis</i> .....	✓	✓	✓	✓	✓
<i>Anemone cylindrica</i> .....	✓	✓	✓	✓	✓	<i>Mesadenia tuberosa</i> .....	✓	✓	✓	✓	✓
<i>Antennaria campestris</i> .....	✓	✓	✓	✓	✓	<i>Onosmodium occidentale</i> .....	✓	✓	✓	✓	✓
<i>Artemisia gnaphalodes</i> .....	✓	✓	✓	✓	✓	<i>Oxalis stricta</i> .....	✓	✓	✓	✓	✓
<i>Asclepias tuberosa</i> .....	✓	✓	✓	✓	✓	<i>Pedicularis canadensis</i> .....	✓	✓	✓	✓	✓
<i>A. verticillata</i> .....	✓	✓	✓	✓	✓	<i>Petalostemon candidus</i> .....	✓	✓	✓	✓	✓
<i>Aster azureus</i> .....	✓	✓	✓	✓	✓	<i>P. purpureus</i> .....	✓	✓	✓	✓	✓
<i>A. laevis</i> .....	✓	✓	✓	✓	✓	<i>Phlox pilosa</i> .....	✓	✓	✓	✓	✓
<i>A. multiflorus</i> .....	✓	✓	✓	✓	✓	<i>Physalis lanceolata</i> .....	✓	✓	✓	✓	✓
<i>A. oblongifolius</i> .....	✓	✓	✓	✓	✓	<i>Psoralea argophylla</i> .....	✓	✓	✓	✓	✓
<i>A. sagittifolius</i> .....	✓	✓	✓	✓	✓	<i>P. esculenta</i> .....	✓	✓	✓	✓	✓
<i>A. sericeus</i> .....	✓	✓	✓	✓	✓	<i>Rosa arkansana</i> .....	✓	✓	✓	✓	✓
<i>Astragalus canadensis</i> .....	✓	✓	✓	✓	✓	<i>Senecio plattensis</i> .....	✓	✓	✓	✓	✓
<i>Baptisia leucophaea</i> .....	✓	✓	✓	✓	✓	<i>Silphium integrifolium</i> .....	✓	✓	✓	✓	✓
<i>Ceanothus pubescens</i> .....	✓	✓	✓	✓	✓	<i>S. laciniatum</i> .....	✓	✓	✓	✓	✓
<i>Chamaecrista fasciculata</i> .....	✓	✓	✓	✓	✓	<i>Sisyrinchium angustifolium</i> .....	✓	✓	✓	✓	✓
<i>Comandra umbellata</i> .....	✓	✓	✓	✓	✓	<i>Solidago glaberrima</i> .....	✓	✓	✓	✓	✓
<i>Coreopsis palmata</i> .....	✓	✓	✓	✓	✓	<i>S. rigida</i> .....	✓	✓	✓	✓	✓
<i>Drymocallis agrimonioides</i> .....	✓	✓	✓	✓	✓	<i>S. rigidiuscula</i> .....	✓	✓	✓	✓	✓
<i>Echinacea pallida</i> .....	✓	✓	✓	✓	✓	<i>Vernonia baldwini</i> .....	✓	✓	✓	✓	✓
<i>Equisetum laevigatum</i> .....	✓	✓	✓	✓	✓	<i>Viola papilionacea</i> .....	✓	✓	✓	✓	✓
<i>Erigeron ramosus</i> .....	✓	✓	✓	✓	✓	<i>V. pedatifida</i> .....	✓	✓	✓	✓	✓
<i>Eryngium yuccifolium</i> .....	✓	✓	✓	✓	✓	<i>Zizia aurea</i> .....	✓	✓	✓	✓	✓

*canadensis*, because of drought, and *Muhlenbergia cuspidata*, a xeric species not thickly distributed in the prairies). Reduction at the third group of stations to nine included the loss of six species by drought (*Andropogon scoparius*, *Carex festucacea*, *Panicum scribneri-*

TABLE 2

BLUESTEM PRAIRIES IN NEBRASKA AND KANSAS, SHOWING SPECIES OF PLANTS OCCURRING AT EACH SAMPLING STATION: 1, LINCOLN; 2, NELSON; 3, HEBRON; AND 4, BELLEVILLE

SPECIES	STATIONS				SPECIES	STATIONS			
	1	2	3	4		1	2	3	4
<i>Agropyron smithii</i> .....	✓	✓	✓	✓	<i>Cirsium undulatum</i> .....	✓	✓	✓	✓
<i>Andropogon furcatus</i> .....	✓	✓	✓	✓	<i>Drymocallis agrimonoides</i> ...	✓	✓	✓	✓
<i>A. scoparius</i> .....	✓	✓	✓	✓	<i>Echinacea pallida</i> .....	✓	✓	✓	✓
<i>Bouteloua curtipendula</i> .....	✓	✓	✓	✓	<i>Erigeron ramosus</i> .....	✓	✓	✓	✓
<i>B. gracilis</i> .....	✓	✓	✓	✓	<i>Hedeoma hispida</i> .....	✓	✓	✓	✓
<i>B. hirsuta</i> .....	✓	✓	✓	✓	<i>Helianthus rigidus</i> .....	✓	✓	✓	✓
<i>Buchloe dactyloides</i> .....	✓	✓	✓	✓	<i>Kuhnia glutinosa</i> .....	✓	✓	✓	✓
<i>Carex festucacea</i> .....	✓	✓	✓	✓	<i>Lepachys columnaris</i> .....	✓	✓	✓	✓
<i>C. pennsylvanica</i> .....	✓	✓	✓	✓	<i>Liatris punctata</i> .....	✓	✓	✓	✓
<i>Eragrostis spectabilis</i> .....	✓	✓	✓	✓	<i>Linum sulcatum</i> .....	✓	✓	✓	✓
<i>Festuca octoflora</i> .....	✓	✓	✓	✓	<i>Lithospermum linearifolium</i> ...	✓	✓	✓	✓
<i>Koeleria cristata</i> .....	✓	✓	✓	✓	<i>Meriolix serrulata</i> .....	✓	✓	✓	✓
<i>Panicum scribnerianum</i> .....	✓	✓	✓	✓	<i>Morongia uncinata</i> .....	✓	✓	✓	✓
<i>P. wilcoxianum</i> .....	✓	✓	✓	✓	<i>Opuntia humifusa</i> .....	✓	✓	✓	✓
<i>Poa pratensis</i> .....	✓	✓	✓	✓	<i>Oxalis violacea</i> .....	✓	✓	✓	✓
<i>Sorghastrum nutans</i> .....	✓	✓	✓	✓	<i>Oxytropis lambertii</i> .....	✓	✓	✓	✓
<i>Sporobolus asper</i> .....	✓	✓	✓	✓	<i>Parosela enneandra</i> .....	✓	✓	✓	✓
<i>S. heterolepis</i> .....	✓	✓	✓	✓	<i>Petalostemon candidus</i> .....	✓	✓	✓	✓
<i>Stipa spartea</i> .....	✓	✓	✓	✓	<i>P. purpureus</i> .....	✓	✓	✓	✓
<i>Acerates angustifolia</i> .....	✓	✓	✓	✓	<i>Physalis lanceolata</i> .....	✓	✓	✓	✓
<i>Allionia linearis</i> .....	✓	✓	✓	✓	<i>Rhus toxicodendron</i> .....	✓	✓	✓	✓
<i>Amorpha canescens</i> .....	✓	✓	✓	✓	<i>Rosa arkansana</i> .....	✓	✓	✓	✓
<i>Anemone caroliniana</i> .....	✓	✓	✓	✓	<i>Senecio plattensis</i> .....	✓	✓	✓	✓
<i>Artemisia gnaphalodes</i> .....	✓	✓	✓	✓	<i>Solidago glaberrima</i> .....	✓	✓	✓	✓
<i>Aster multiflorus</i> .....	✓	✓	✓	✓	<i>S. rigida</i> .....	✓	✓	✓	✓
<i>Baptisia leucophaea</i> .....	✓	✓	✓	✓	<i>Vernonia baldwini</i> .....	✓	✓	✓	✓

*anum*, *P. wilcoxianum*, *Poa pratensis*, and *Sorghastrum nutans*). *Bouteloua hirsuta*, *Eragrostis spectabilis*, and *Sporobolus heterolepis* formerly occurred, if at all, only sparingly. No new species were gained. A final reduction to only four grasses at the western Kansas stations resulted from death of all the mid grasses which formerly occurred there except *Bouteloua curtipendula*.

Among the grasses, *B. curtipendula* alone was found at all four groups of stations, thus illustrating its wide adaptability and drought-resistant qualities. Conversely, *Stipa spartea* occurred only at the first two, but the deeply rooted *Andropogon furcatus* and the rapidly reproducing *Koeleria cristata* at all but the last. *Buchloe dactyloides* and *Bouteloua gracilis*, most xeric of all, occurred at all three western

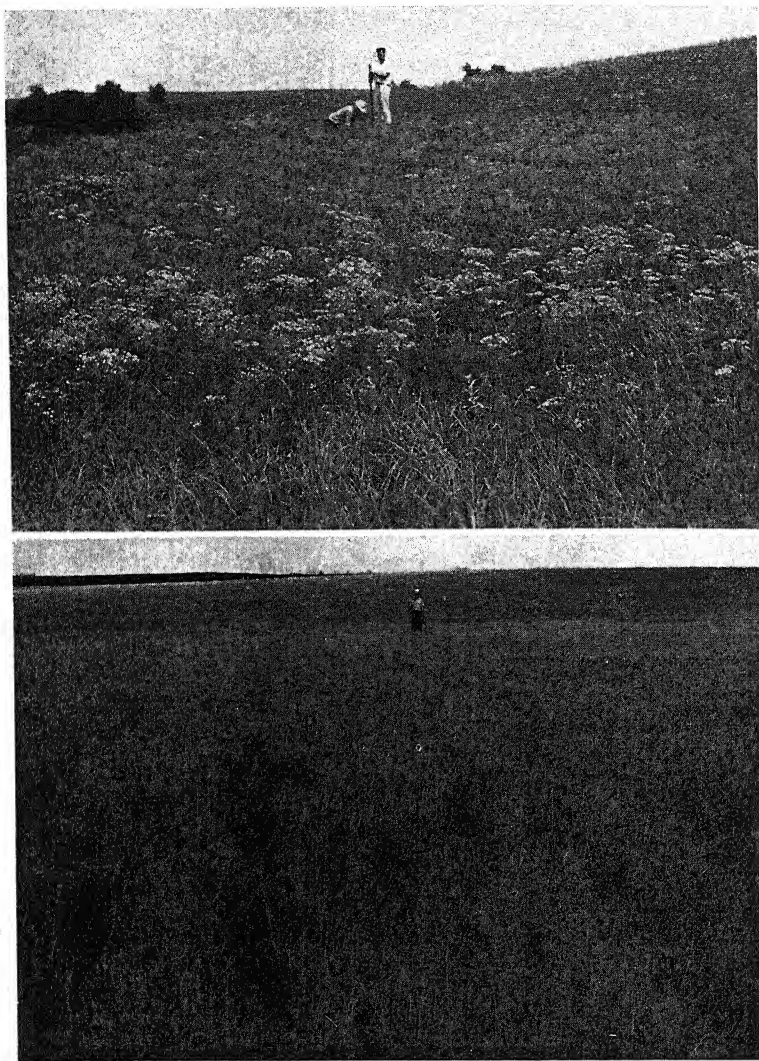
TABLE 3

WHEAT-GRASS PRAIRIES IN NEBRASKA AND KANSAS, SHOWING SPECIES OF PLANTS OCCURRING AT EACH SAMPLING STATION: 1, CLAY CENTER; 2, CARLETON; AND 3, MONTROSE

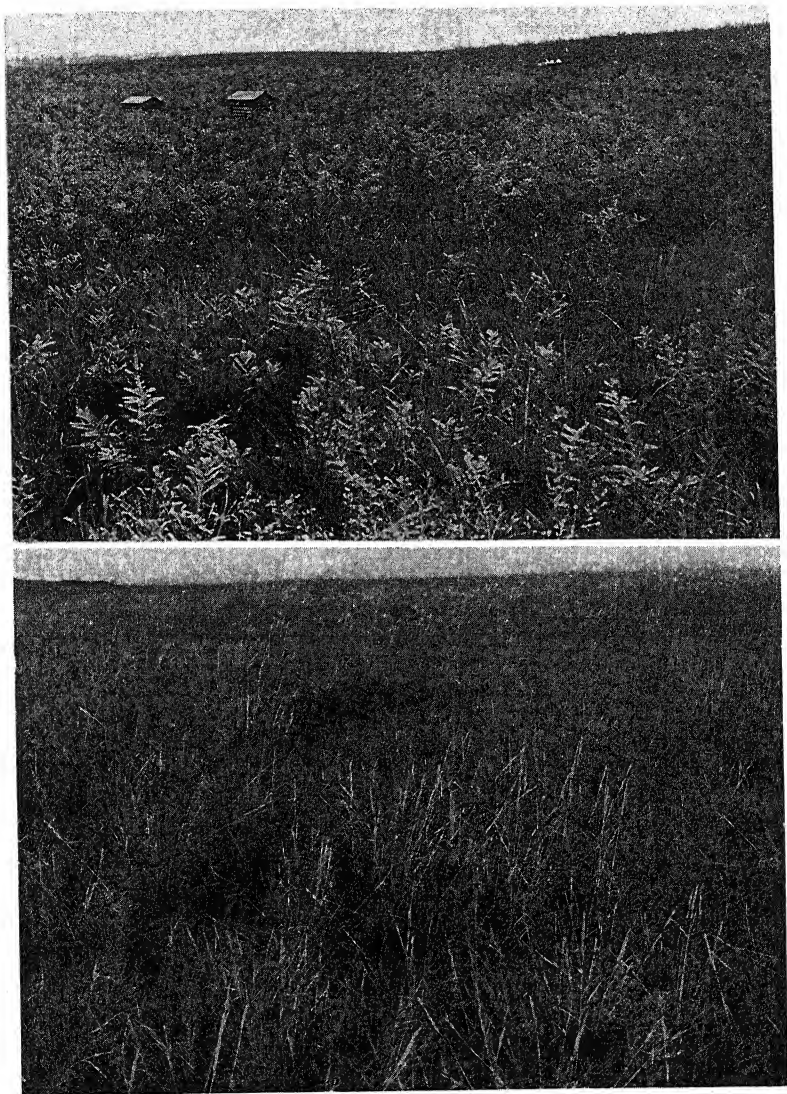
SPECIES	STATIONS			SPECIES	STATIONS		
	1	2	3		1	2	3
<i>Agropyron smithii</i> .....	✓	✓	✓	<i>Cirsium undulatum</i> .....	✓	...	...
<i>Andropogon furcatus</i> .....	...	✓	✓	<i>Kuhnia glutinosa</i> .....	✓	✓	✓
<i>Bouteloua curtipendula</i> .....	✓	✓	✓	<i>Liatris punctata</i> .....	✓	✓	...
<i>B. gracilis</i> .....	✓	✓	✓	<i>Lygodesmia juncea</i> .....	✓	...	✓
<i>Buchloe dactyloides</i> .....	...	✓	✓	<i>Malvastrum coccineum</i> .....	✓	...	...
<i>Carex pennsylvanica</i> .....	✓	✓	✓	<i>Meriolix serrulata</i> .....	✓	✓	...
<i>Festuca octoflora</i> .....	...	✓	...	<i>Oxalis violacea</i> .....	✓	✓	✓
<i>Koeleria cristata</i> .....	✓	✓	...	<i>Physalis lanceolata</i> .....	...	...	✓
<i>Sporobolus asper</i> .....	...	✓	...	<i>Rosa arkansana</i> .....	...	✓	✓
<i>Acerates angustifolia</i> .....	✓	✓	✓	<i>Salvia pitcheri</i> .....	...	...	✓
<i>Allionia linearis</i> .....	...	...	✓	<i>Senecio plattensis</i> .....	✓	...	...
<i>Amorpha canescens</i> .....	✓	✓	✓	<i>Sideranthus spinulosus</i> .....	...	...	✓
<i>Artemisia gnaphalodes</i> .....	✓	...	...	<i>Solidago glaberrima</i> .....	✓	...	...
<i>Asclepias verticillata</i> .....	✓	...	...	<i>S. mollis</i> .....	✓	...	✓
<i>Aster multiflorus</i> .....	✓	✓	✓	<i>Vernonia baldwini</i> .....	...	...	✓

groups of stations; in fact, they are rapidly spreading to replace mid grasses.

Reduction of forbs from sixty-five in Iowa to thirty-three in the Lincoln group occurred notwithstanding the gain of a total of nine species, including the xeric *Allionia linearis*, *Cirsium undulatum*, *Opuntia humifusa*, and *Oxytropis lambertii*. The further reduction to twenty-one species was accompanied by the addition of three western ones—*Malvastrum coccineum*, *Sideranthus spinulosus*, and *Solidago mollis*. The final group of only eleven perennial forbs includes two xeric species (*Psoralea tenuiflora* and *Allium nuttallii*) not found in the preceding sampling areas.



FIGS. 14, 15.—Fig. 14 (above), prairie at Anita, Iowa, August 15, 1939, showing abundance of forbs, most conspicuous of which is *Euphorbia corollata*. Fig. 15 (below), prairie near Lincoln, Nebraska, August 1, 1939, showing almost entire absence of forbs, typical of conditions at all but the Iowa stations.



FIGS. 16, 17.—Fig. 16 (above), portion of Belmont prairie at Lincoln, July 6, 1932, showing abundance of *Amorpha canescens* and other forbs in cover of *Andropogon scoparius*. Fig. 17 (below), same area on July 6, 1939. Note great decrease in forbs and replacement of little bluestem by *Stipa spartea* and other more xeric grasses.

Six species of the ground layer or understory occurred only at the most mesic group of stations, although they were formerly plentiful in the Lincoln group also, some species being common even farther westward. These are *Antennaria campestris*, *Sisyrinchium angustifolium*, *Viola pedatifida*, *V. papilionacea*, *Fragaria virginiana*, and the rosettes of *Hieracium longipilum*. Clearly this depletion of the ground layer was an effect of drought. Three other understory species (*Carex pennsylvanica*, *Oxalis violacea*, and *Anemone caroliniana*) not only persisted but increased in abundance, probably because of underground storage organs (4).

A number of very deeply rooted species occurred at all but the most xeric group of stations. These were *Amorpha canescens*, *Rosa arkansana*, *Vernonia baldwini*, *Solidago glaberrima*, and *Aster multiflorus*. *Senecio plattensis* and *Artemisia gnaphalodes* accompanied them. *Kuhnia glutinosa*, which is very drought resistant, probably because of its excellent, deeply seated taproot, was the only species found at all four station-groups. In fact, it occurred at each of eleven stations, although never abundantly. Other species found at the three more xeric groups of stations, and which are notably drought resistant, are *Liatris punctata*, *Cirsium undulatum*, *Allionia linearis*, and (absent in the Lincoln group) *Lygodesmia juncea*. The equally xeric *Malvastrum coccineum* and *Sideranthus spinulosus*, found only at the two driest station-groups, are not naturally distributed so far eastward as the preceding.

It is of interest and importance that of the eleven Leguminosae in Iowa, the number was reduced first to seven, and then to one at each of the more westerly station-groups. The much more regular occurrence of many species at all the stations in the Iowa group, unaffected by drought, is also significant. Of the total of fifteen grasses or grasslike species, thirteen were found at each station, but only ten to thirteen of a total of nineteen at each station of the Lincoln group.

These data, presented from the relatively small but numerous sampling areas, are in general substantiated by much more extensive surveys, including entire prairies, which are now under way. They indicate clearly some of the drastic changes in plant populations resulting from the years of drought. A loss of 50-66 per cent

of forbs was found in 1938; during 1939 they have been further depleted both in numbers and species.

The value of this study of environmental relations, density of plant cover, distribution of forbs, etc.—although done intensively only in this central area of the mid-continental grasslands—is enhanced by the fact that it represents generally the great changes that have been wrought in the grasslands from east to west by the several years of drought.

### Summary

1. Four groups of prairies of known previous history, extending from southwestern Iowa to western Kansas, were studied in 1939.

2. The Iowa group (precipitation about 32 inches) had been scarcely affected by the great drought. At one Nebraska-Kansas group, bluestem grasses (*Andropogon* spp.) still prevailed; at the other they had died and been replaced by *Agropyron smithii*. *Bouteloua gracilis* and *Buchloe dactyloides* were the chief grasses of the western Kansas prairies (precipitation about 22 inches).

3. The twelve prairies in the four groups all occurred on deep, fertile, silt-loam soils; water content was determined monthly to a depth of 6 feet.

4. The bluestem prairies of Iowa, where rainfall was plentiful, had a continuous supply of available water at all depths; those of Nebraska and Kansas had only a low supply after early spring and were repeatedly threatened with drought. In the wheat-grass group, available deep-soil moisture was the exception, and repeated exhaustion of surface soil moisture occurred. Water was available only in the surface soil at the short-grass stations and at only two or three periods.

5. Slightly higher temperatures, 50–100 per cent more evaporation, and two to three times as much wind movement occurred in western Kansas as in eastern Nebraska.

6. No previous deterioration of vegetation had occurred in Iowa. The ground layer had been destroyed and the basal area was only one-half to two-thirds normal in the drought-depleted bluestem prairies westward. Drought and dust had destroyed most of the former plant cover of the third group of prairies, which were now

dominated by an open growth of western wheat grass which permitted a continuous pattern of bare soil. In western Kansas the 85 per cent basal cover of short grasses had been reduced by continued drought, burial by dust, and injury by grasshoppers to 10-15 per cent. The remaining soil was bare.

7. Grasses grew normally in Iowa, reaching a foliage level of 18 inches; they dried after midsummer and failed to flower in eastern Nebraska. Wheat grass dried very early and burned readily in July. The short grasses in west-central Kansas were dormant during most of the summer but grew to a height of 3 inches when revived by late summer showers.

8. In Iowa, fifteen species of grasses and sedges occurred at the sampling stations, but nineteen at the western, bluestem station-group. There were but nine at the wheat-grass stations and only four in the short-grass areas.

9. Native forbs similarly decreased from sixty-five to thirty-three species, then to twenty-one, and finally to eleven.

10. Conditions of the central area of the mid-continental grassland reveal in general changes that have been wrought elsewhere from east to west by continued drought.

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## SIZE OF NUCLEI IN THE SHOOT OF RICINUS COMMUNIS

FLORA MURRAY SCOTT

(WITH SEVEN FIGURES)

### Introduction

While working on the differentiation of the spiral vessels in *Ricinus* (2), the extremely large size of the nuclei within the coenocytes was noticed. It was decided to examine further the question of nuclear size in the shoot as a whole. MONSCHAU (1) has shown that, in the case of certain typical monocotyledons and dicotyledons, the nucleus of parenchyma cells such as pith and cortex measures approximately eight times the volume of the meristem nucleus. Statistical evidence given in this paper indicates that volume increase is effected by doubling and redoubling of nuclear material.

In *Ricinus* the volume of the meristem nuclei is approximately 38 cu.  $\mu$ , that of the majority of nuclei of pith and cortex 332 cu.  $\mu$ , an eightfold increase. In marked contrast to this, the largest nuclei of the developing spiral vessels, and also of the secondary vessel segments, may measure as much as 59,000 cu.  $\mu$ , or 1550 times the original nuclear volume.

In the present paper a survey is made of the nuclear volume in the shoot as a whole, with particular stress on the "giant" nuclei of spiral elements and of the pitted vessel segments of the primary and secondary tissues, respectively, in axis and in petioles. Certain preliminary evidence in regard to the nature of the components responsible for volume increase—chromatin, nucleolus, karyolymph, and water—is also presented.

### Material and methods

Shoot tips and secondary tissues of actively growing plants of *Ricinus communis* were fixed in Navashin's solution, imbedded, sectioned, and stained either with haematoxylin or with safranin and fast green. Sections were necessarily cut 10–60  $\mu$  thick, depending on the region of the axis and the vessel diameter. Measurements

were made both of fresh material and of fixed preparations. The nuclei are spherical or ellipsoidal, and volumes are calculated from the formulas  $\frac{4}{3}\pi r^3$  and  $\frac{4}{3}\pi a^2b$ .

In determining nuclear components, mitotic figures were examined in coenocytes and in meristematic tissues. The varying volumes of the nucleoli were noted in relation to the size of the containing nuclei. The relative amount of water present in larger and smaller nuclei was estimated by the volume contraction induced by irrigation with absolute alcohol, followed or not by xylene.

### Observations

#### VOLUME OF NUCLEI OF SPIRAL ELEMENTS AND DIAMETERS OF SPIRAL VESSELS

The spiral elements, like other primary vascular cells, differentiate from procambial tissue but pass through a characteristic coenocytic phase, the details of which have been previously described (3).

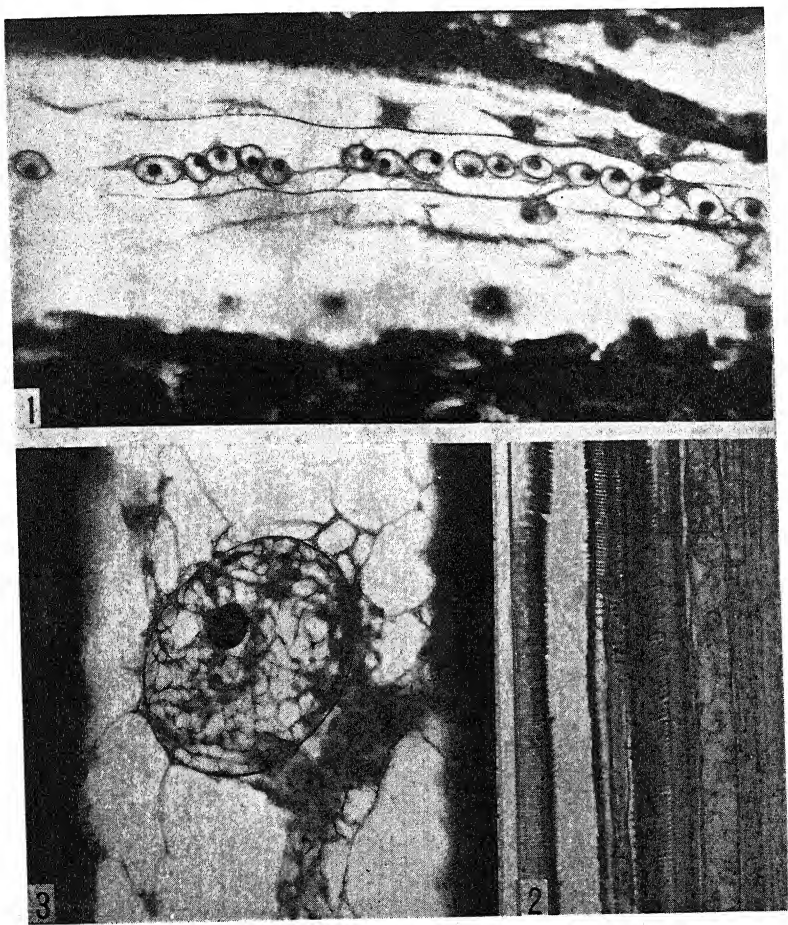
TABLE 1

NUCLEAR VOLUMES (IN ROUND FIGURES) OF SPIRAL VESSELS IN  
1000 CU.  $\mu$  GROUPS AND THEIR FREQUENCIES

Volume.....	0-999	1000	2000	3000	4000	5000	6000	7000	8000	9000
Frequency....	570	313	125	71	31	21	17	17	8	8
Volume.....	10,000	11,000	12,000	13,000	14,000	16,000	17,000	18,000	20,000	30,000
Frequency....	8	5	5	1	4	1	4	1	4	1
Volume.....	40,000	50,000								
Frequency....	1	1								

Coenocytes may show from two to seventeen or more nuclei (fig. 1). The nuclei persist after the spiral thickening is laid down (fig. 2). Nuclear measurements are made in the developing coenocytes in all stages of development.

Nuclear volumes increase in passing from younger to older internodes. In the shoot as a whole the volume measurements form a continuous series from 38 to 59,000 cu.  $\mu$ . Certain volumes, however, occur more frequently than others. The range of nuclear volume in the petiole is similar to that in the shoot. When volumes are arranged in size groups of 1000 cu.  $\mu$ , it is seen that the greatest number of nuclei do not exceed 2000 cu.  $\mu$  in volume (table 1). When the volumes are plotted in detail, however, up to 4000  $\mu$  a many-peaked graph is the result (table 2 and fig. 4). The maximal frequencies in



FIGS. 1-3.\*—Fig. 1, longisection of stem apex showing multinucleate coenocyte of differentiating spiral vessel. Fig. 2, older internode showing coenocyte adjacent to lignified vessels, protoplast with nuclei present in one of the latter. Fig. 3, same showing reticulum of nucleus, nucleolus, nuclear sap, etc., in "giant" nucleus, adjacent to fully lignified vessel.

\* Since only one optical level in a section can be accurately focused at any one time, details of protoplasts of photomicrographs are outlined for the sake of greater clearness.

the first section of the graph occur at 332, 585, 755, 1146, and 1360 cu.  $\mu$ . Thereafter with increasing volume the lesser maxima appear

TABLE 2  
NUCLEAR VOLUMES IN SPIRAL VESSELS IN INTERNODES AND IN  
PETIOLES (IN CU.  $\mu$ ) AND THEIR FREQUENCIES (ABBREVIATED); 1212 MEASUREMENTS

VOLUMES 38-4000											
Volume.....	332	385	755	1073	1146	1360	1640	2087	2743	3159	3953
Frequency.....	94	36	78	34	55	70	33	17	18	14	9
VOLUMES 5000-10,000 (in round figures)											
Volume.....	± 5500		± 6500		± 7500		± 8500		± 9500		± 10,500
Frequency.....	21		17		17		8		8		8
VOLUMES 11,000-20,000											
Volume.....	± 11,500		± 12,500		± 13,500		± 14,500		± 16,500		± 17,500
Frequency.....	5		5		1		3		2		3
VOLUMES 20,000-30,000											
Volume.....	± 20,000		± 22,000		± 24,000		± 26,000		± 28,000		± 30,000
Frequency.....	1		1		1		1		1		1
VOLUMES 30,000-60,000											
Volume.....	32,013		48,398		57,226		59,550				
Frequency.....	1		1		1		1				

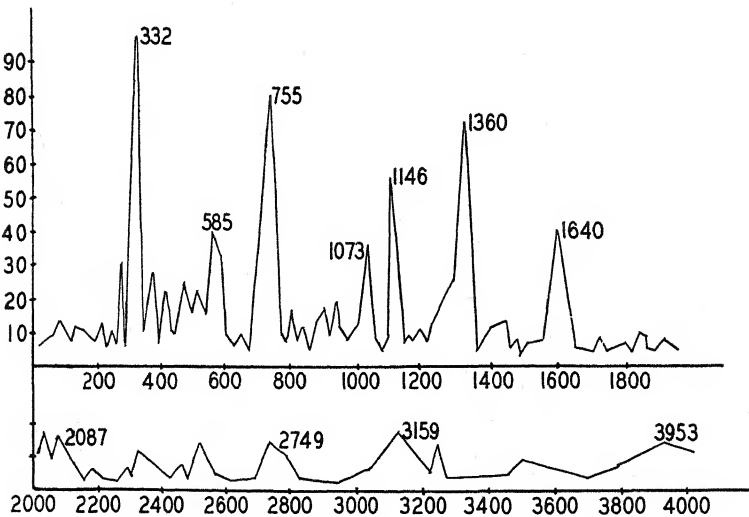


FIG. 4.—Volumes of spiral vessel nuclei of internodes and petioles (32-4000 cu.  $\mu$  only) and their frequencies (cf. table 2).

with less regularity, and, if continued above 5000 cu.  $\mu$ , the curve would flatten to reach the maximum volume of 59,550, a 1550-fold increase. The decreasing numbers in this region are due in part to greater technical difficulties in dealing with thicker sections.

The nuclei of the differentiating vessels are spherical or ellipsoidal. The diameters or minor axes of the nuclei of maximum frequency are given in table 3, and are followed by those of the larger nuclei, which are fewer in number.

The nuclear diameters of all nuclei thus range from 6 to 35, and the diameters of the volumes up to 3000 are (in round numbers): 6, 7, 9, 10, 13, 17.

TABLE 3

VOLUME	DIAMETER ( $\mu$ )	VOLUME	DIAMETER ( $\mu$ )
NUCLEI OF MAXIMUM FREQUENCY			
332	8.7	2749	17.4
585	10.4	3159	13.0
755	6.9	3261	8.7
1146	13.0	3511	13.0
1360	8.7	3953	10.4
1640	10.4	4210	17.4
2048	13.0	4937	13.0
2087	8.7	5405	11.3
2530	10.4		
LARGER NUCLEI			
6237	13.0	15,349	15.6
7900	16.5	17,598	21.7
9873	17.4	33,544	17.4
12,936	27.8	59,550	34.8

When nuclei are measured *in situ* it is seen that the diameter of the largest measures slightly more than half that of the containing vessel or coenocyte. In the largest vessels, 70-180  $\mu$  diameter, this ratio is not sustained. The increase in volume of the largest ellipsoidal nuclei is due to increase along the major axis.

Since nuclei are spherical or ellipsoidal, it is apparent that their volumes in the same coenocyte, and in different coenocytes of the same diameter, may vary widely. The arrangement of larger and smaller nuclei within a single coenocyte appears to be haphazard (table 5). There is no gradation in size from one end of the coenocyte to the other.

## NUCLEI OF SECONDARY VESSEL SEGMENTS

The vessel segments of the secondary xylem measure 250-400  $\mu$  in length and 60-110  $\mu$  in width. The perforated end walls are generally

TABLE 4

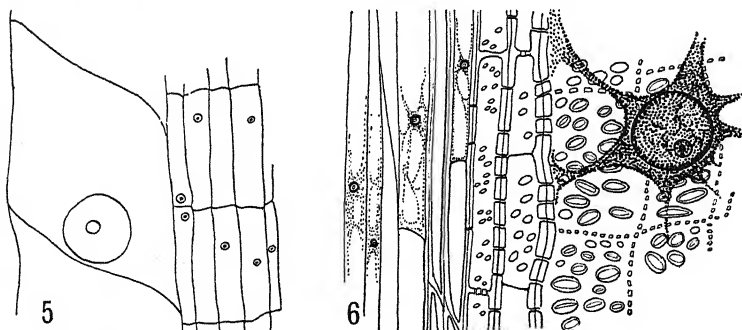
DIAMETERS OF NUCLEI IN RELATION TO VESSELS; 217 MEASUREMENTS

Vessel diameter ( $\mu$ ).....	15	17	21	26	30	34	39	44	52	60
Range of nuclear diameters....	6-10	6-11	8-12	8-13	8-17	8-18	9-20	11-26	13-26	17-30

TABLE 5

VARIATIONS OF NUCLEAR VOLUMES IN SAME COENOCYTES; NUCLEI  
LIE ADJACENT IN PLASMOLYZED PROTOPLASTS

1	2	3	4	5
585	755	755	383	816
3050	2530	483	1360	755
1146	2087	332	483	3201
483	732	585	560	291
2749	332	613	755	1073
			430	1073
			358	



FIGS. 5, 6.—Fig. 5, longisection of secondary xylem (camera lucida) showing contrast between nuclear size in cambium and in differentiating vessel segment. Fig. 6, same showing border-pitted wall in lignified vessel segment; protoplast still present.

steeply oblique, and the vertical walls are heavily pitted with bordered, or occasionally simple, pits (figs. 5, 6). In a single longitudinal radial section it is possible to observe both cambial and vessel nu-

clei. The latter have a range in volume equal to that found in the spiral vessels. Volumes of 7629, 16,428, 22,075, 25,196, 55,351 are among those recorded. The former, the cambial nuclei, retain the initial volume of the meristem nuclei, 38 cu.  $\mu$ .

#### NUCLEI OF PARENCHYMA

In the first 20 cm., approximately twelve to fifteen internodes, the nuclei of the pith are readily observed. Below this point starch-containing plastids form an envelope around the nucleus and obscure accurate definition. In the younger internodes the nuclei range from

TABLE 6  
VOLUMES OF PITH NUCLEI AND THEIR FREQUENCIES

0-99	100	200	300	400	500	600-999	1000	2000	3000	4000
38-7	100-3	215-1	312-1	407-1	560-1	656-1	1146-25	2058-2	3084-2	4180-3
58-1	112-29	230-1	332-53	430-14	585-23	732-12	1314-1	2200-2		
73-35	124-4	237-1	358-1	483-15		755-1		2394-1		
97-4	133-1	247-2	383-3			772-2		2404-4		
	144-4	261-5				902-2		2620-1		
	164-38	275-1				918-4		2749-2		
		299-1						2848-1		

38 to 332 cu.  $\mu$  in volume. In the older internodes the volume may reach 4000 cu.  $\mu$  (table 6).

Plotting of volume-frequency from a more limited number of measurements (300) results, as in the case of the vessel nuclei, in a many-peaked graph, with maxima at 73, 164, 332, and lesser maxima at 112, 585, and 1146 (fig. 7). If the volume of the meristem nucleus, 38 cu.  $\mu$ , is regarded as unit volume =  $x$ , then the first three maxima appear as approximate multiples of this figure,  $2x$ ,  $4x$ ,  $8x$ , the actual multiples being 76, 153, 304. This is in agreement with the findings of MONSCHAU (2). The lesser maxima, forming what is essentially a continuous series, represent approximate multiples— $3x$ ,  $15x$ ,  $30x$ . The largest nuclei occur in the older internodes and range from 500 to 4000 cu.  $\mu$ , but thus do not approach the volume of the largest vessel nuclei. The nuclei of cortex, rays, and other tissues lie within the same general range as the nuclei of the pith.

## NUCLEOLUS

The nucleolus varies relatively little in size and does not keep pace with the rapidly enlarging nucleus. While the nucleus increases from 38 to 3000 cu.  $\mu$ , the nucleolus does not change appreciably from its initial volume, 19 cu.  $\mu$ . Thereafter as the nucleus reaches 9000, 17,000, and 33,000 cu.  $\mu$ , the nucleolus measures 71, 330, and 943. In the largest nuclei, those over 10,000 cu.  $\mu$ , there is no constant relation between nuclear and nucleolar size. This ratio may range erratically from 35:1 to 298:1 (table 7).

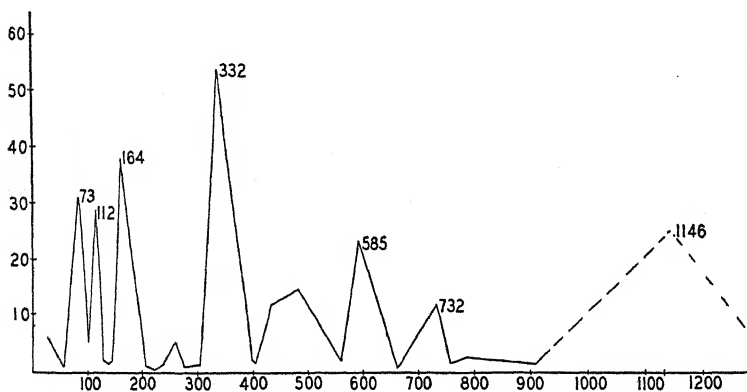


FIG. 7.—Volumes of nuclei of pith and their frequencies (30-1200  $\mu$  only)

## CYTOLOGY OF NUCLEI

An important question in regard to the giant nuclei is, To what material is due the enormous increase in bulk—chromatin, nucleolus, karyolymph, or water? (fig. 3).

I. CHROMATIN.—In meristematic nuclei the number of chromosomes observed is nineteen. In developing coenocytes a certain number of mitotic divisions has been observed in metaphase and anaphase condition. The number of chromosomes has not increased, therefore the increase in volume is not attributable to a polyploid condition. The size of the individual chromosomes appears to remain constant, and the bulk of chromatin material as a whole when measured in anaphase is not significantly greater. Increased volume therefore does not depend on increased chromatin.

2. NUCLEOLUS.—The size of the nucleolus has already been discussed and its variability in larger nuclei noted. The nucleolus does not contribute significantly to increased nuclear volume.

3. KARYOLYMPH, WATER.—The addition of absolute alcohol to fresh material causes marked contraction in nuclear size. The addi-

TABLE 7  
RATIO OF VOLUME OF NUCLEUS TO VOLUME  
OF NUCLEOLUS

VOLUME OF NUCLEUS	VOLUME OF NUCLEOLUS	RATIO
332	19	16:1
585	19	28:1
755	19	39:1
1300	19	71:1
1360	38	35:1
2087	19	109:1
2087	38	54:1
2530	19	133:1
2530	38	66:1
3159	38	83:1
4937	38	127:1
9126	71	128:1
10,569	196	54:1
10,731	71	151:1
11,051	37	298:1
11,499	58	198:1
11,895	163	72:1
12,617	112	112:1
14,157	96	147:1
14,637	259	56:1
17,599	330	53:1
34,544	943	35:1

tion of xylene does not induce further shrinkage. It is assumed that water is the only component removed from the nucleus in this dehydration process. Whether the contraction indicates the total volume of free water remains an open question. The nucleolus does not change appreciably in volume in the nuclei observed. Shrinkage in volume on dehydration varies in meristematic and in differentiated nuclei, as shown in tables 8 and 9.

In meristem cells between which intercellular spaces have not yet developed, the contraction is approximately 42 per cent. In the nuclei of the meristematic dividing region, intercellular spaces ap-

pear and vacuolation of cells has begun. Shrinkage ranges from 27 to 81 per cent. In the pith and spiral vessel nuclei the same variability in contraction is evident. The spiral vessel nuclei show a fairly consistent increase in water loss with increasing size.

TABLE 8  
CONTRACTION OF NUCLEI ON ADDITION OF ABSOLUTE ALCOHOL

MATERIAL	ORIGINAL VOLUME* (cu. $\mu$ )	VOLUME AFTER CONTRACTION (cu. $\mu$ )	PERCENTAGE	
			CONTRACTION	RESIDUAL VOLUME (cu. $\mu$ )
Stem apex: meri- stematic dividing cells; no vacuoles present	136	81	40	60
	112	65	41	59
	112	65	41	59
	94	52	43	57
Stem apex: vacuo- lating dividing cells; intercellular spaces developing	112	65	41	69
	112	65	41	69
	112	65	41	69
	65	33	47	53
	65	33	47	53
Stem apex: same as preceding	372	270	27	33
	687	425	38	62
	410	171	58	42
	522	94	81	19
Pith of eleventh and thirteenth inter- nodes	522	233	55	45
	1146	522	54	46
	1761	1146	54	46
	1879	1337	28	72
	2280	1761	22	78
	2555	1526	40	60
	5543	2140	61	39
	5543	1761	68	32

\* Nucleolus included in the volumes.

These preliminary findings indicate that increase in volume is accounted for to a great extent by increased water content. The remaining increase in size over and above that accounted for by water must therefore be attributed to karyolymph. Compare, for example, the nuclei of volumes 10,344 and 51,128 with their volumes after dehydration, 2056 and 9202. The smallest meristematic nuclei measure (minus the nucleolus) 34 cu.  $\mu$ . If the water content of such a nucleus is assumed to be 40 per cent, then the volume of karyolymph

and chromatin measures 22 cu.  $\mu$ . In the nuclei under discussion a 200-fold and a 400-fold increase in the karyolymph volume is therefore apparent.

#### TIME OF MITOTIC DIVISION

The time of mitotic division is undetermined. Since young coenocytes occur with as many as sixteen to twenty-two small nuclei of

TABLE 9  
CONTRACTION OF NUCLEI CONTINUED

MATERIAL	ORIGINAL VOLUME (cu. $\mu$ )	VOLUME AFTER CONTRACTION (cu. $\mu$ )	PERCENTAGE	
			CONTRACTION	RESIDUAL VOLUME (cu. $\mu$ )
Spiral vessel nuclei	253	119	52	48
	253	119	52	48
	570	344	39	61
	738	266	63	37
	738	103	86	14
	1148	266	76	24
	1148	266	76	24
	1226	344	71	29
	1226	344	71	29
	1315	266	79	21
	2780	738	73	27
	6025	3861	49	51
	7642	1894	68	32
	10,344	2056	80	20
	12,302	4460	64	36
	27,254	4961	81	18
	51,128	9202	82	28

Volume of nucleolus subtracted in the volumes.

identical size adjacent in the same protoplasmic strand, it is presumed that nuclear division takes place repeatedly in the earliest stages of differentiation (fig. 1); but mitotic division is also occasionally observed in coenocytes of larger diameter.

The preliminary data on nuclear contraction already cited thus indicate that meristematic activity may be definitely linked with limited water content.

#### Summary

1. During differentiation of the primary spiral vessels of *Ricinus communis* the nuclei of the coenocytes become extremely large. The volume of typical meristem nuclei is 38 cu.  $\mu$ , that of spiral vessel

nuclei may range up to 59,000 cu.  $\mu$ , a 1550-fold increase in volume. The nuclei of maximal frequency do not fall into the size groups of MONSCHAU, K<sub>1</sub>:K<sub>2</sub>:K<sub>3</sub>:K<sub>4</sub> of volume ratios 1:2:4:8, in which each class is a multiple of the preceding. Nuclear and vessel diameter are interdependent except in the largest vessels. The nuclei attain their maximum size at the beginning of the process of spiral thickening, remaining intact and presumably functional for some time after lignification is complete.

2. In the younger internodes of the pith the nuclei fall into a series approximating MONSCHAU'S size groups, with a maximum frequency at 332 cu.  $\mu$ , an eightfold volume increase. In older internodes they range in size up to 4000 cu.  $\mu$ .

3. In regard to the material responsible for nuclear size, it is seen that: (a) In mitotic divisions the nucleus remains diploid and the chromosomes are not significantly greater than those of the meristem. (b) The nucleolus does not keep pace with the increasing size of the nuclei, and in the largest nuclei the nucleolar-nuclear ratio is highly variable. (c) Karyolymph increases 200- to 400-fold in volume. Water removable by the addition of absolute alcohol may constitute as much as 80 per cent of nuclear volume. Increase in volume is therefore attributable to these two factors.

4. Meristematic activity and nuclear water content appear to be interdependent.

5. It seems improbable that the vessel nuclei of *Ricinus* are unique in their nucleo-cytoplasmic ratio. Re-examination of thicker sections of fresh and fixed material of other large-vesselled plants may reveal conditions similar to that in *Ricinus*.

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# GROWTH RESPONSES OF BILOXI SOYBEANS TO VARIATION IN RELATIVE CONCENTRATIONS OF PHOSPHATE AND NITRATE IN THE NUTRIENT SOLUTION

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 510

CHARLES L. HAMNER

(WITH FOUR FIGURES)

## Introduction

Experimental results from many nutrient experiments have shown that, in general, if an element is lacking or low in concentration in the nutrient solution or soil solution, it will be lacking or present only at very low concentrations in the plant. Also, an essential element present in abundance in the nutrient solution will probably be present in abundance in the plant, although its concentration may vary with variation in environmental factors. A factor which may affect the accumulation of one element in the plant is the presence or absence of another essential element in the nutrient solution (2). The deficiency of an element or its abundance in the nutrient solution may affect the rate and amount of absorption of other elements, and thus affect growth responses.

It is probable that for every plant there is a specific nutrient requirement for maximum vegetative expression, flowering, fruit production, etc., and it is probable that this requirement changes as the conditions within the plant or external environments change. Just what constitutes optimum requirements will depend upon the viewpoint of the investigator, but many workers have utilized vegetative growth responses as a measure of the suitability of any particular nutrient solution. Two methods of measuring the growth responses of plants are possible, and may be classified as qualitative and quantitative. Quantitative measurements are concerned with the total accumulation of dry matter by the plant; qualitative measurements are concerned with the character or types of material composing the plant—proteins, fats, vitamins, etc. After a definite corre-

lation between quantitative response and nutrient requirements has been established, then an investigation of the qualitative responses may lead to an understanding or interpretation of the quantitative responses.

The object of the present study was a determination of the relationships existing between growth responses and the concentration of various ions in sand and solution culture. Since it is impossible to measure and record all the growth responses of any particular group of plants, and since the summation of the qualitative responses represent the quantitative responses, the criteria of response used here have been the accumulation of dry matter by the plant and its parts and the general appearance of the plant.

### Experimental methods

Biloxi soybeans were used as experimental material. Several preliminary experiments were carried out, both in sand and solution culture, but the detailed results presented have been taken only from sand culture experiments, since plants grew as well in sand culture as in comparable solution cultures and the former experiments were more readily carried out. In all experiments, seeds were selected for uniformity and planted at an even depth in well-washed white quartz sand. They were then placed in darkness or in the greenhouse and watered with tap water. After germination, uniform seedlings were selected and transplanted to quartz sand contained in glazed 8×4-inch crocks with a  $\frac{3}{8}$ -inch hole in the bottom, each crock containing about 1500 cc. of sand. They were maintained on a well lighted greenhouse bench. After transplantation, the plants were irrigated with the various nutrients. The preliminary experiments were carried out in the early summer when the length of a natural day was in excess of the critical photoperiod (4). The final experiments were carried out in the late summer and the plants received supplementary illumination of an intensity of about 20 foot-candles from sunset to 2 A.M., so that the plants remained vegetative throughout the experimental period. No attempt was made to control greenhouse temperatures, although excessively high temperatures were avoided by careful ventilation. Excessively low atmospheric humidity was prevented by frequent spraying of the walks

and walls with water during warm dry days. The plants were watered each day with sufficient nutrient solution so that an excess dripped out of the openings in the bottom of the crocks; twice a week the pots were thoroughly flushed with distilled water, immediately followed by application of fresh nutrient solution.

### HARVESTING

In preliminary experiments, observations were made on the effect of an increase in the concentration of a single one of the major essential elements of the nutrient solution by adding its sodium salt or its chloride, depending upon whether the element under consideration was an anion or a cation. The procedure was to use a three salt solution (8), which promotes fairly active vegetative growth, but to use this at about one-fifth the concentration ordinarily employed. Growth of plants in this solution was compared with the growth obtained by adding various single salts to it.

The various nutrient solutions used and the results obtained are shown in table 1. The experiment was continued for 16 days after transplanting.

The result of the preliminary experiment showed that with a concentration one-fifth that of the complete nutrient solution used, or even with one-tenth concentration of the complete solution, active vegetative growth could be obtained without any deficiency symptoms appearing. The complete solution diluted to one-fifth concentration, to which KCl had been added, showed the greatest growth next to the complete solution. The complete solution diluted to one-fifth, to which  $\text{NaH}_2\text{PO}_4$  had been added, showed evidence of toxicity. The complete solution diluted to one-fifth, to which NaCl had been added, also showed poor growth (table 1). It was not possible to tell whether the decreased growth was caused by Na or by Cl or both. It was also not possible to decide whether the toxicity was caused by Na or by the  $\text{HPO}_4$ .

Because of the difficulty of interpreting the results of the preliminary experiments, another method was utilized in making up the nutrient solutions. By this procedure two series of nutrient solutions were obtained. In one series the cations (Ca, Mg, and K) would vary in relation to one another while the anions (S, N, and P)

remained constant throughout the series. In the other, the anions varied in relation to one another while the cations remained constant. Thus two nutrient triangles, similar in some ways to those presented by SHIVE (8), were utilized. The advantage of this system was that the concentration of only three ions varied from square to square in any given triangle (fig. 1), while in SHIVE's system there

TABLE 1

EFFECT OF ADDITION OF SINGLE SALTS TO STANDARD THREE SALT  
NUTRIENT SOLUTION ON GROWTH OF BILOXI SOYBEANS  
(PER 100 PLANTS)

COMPOSITION OF NUTRIENT SOLUTION	TOTAL DRY WEIGHT (GM.)	DRY WEIGHT OF TOPS	DRY WEIGHT OF ROOTS	TOP/ROOT RATIO
A. Complete solution $\left\{ \begin{array}{l} \text{MgSO}_4 \text{ } 0.0045 \text{ mol} \\ \text{Ca(NO}_3)_2 \text{ } 0.0060 \text{ mol} \\ \text{KH}_2\text{PO}_4 \text{ } 0.0045 \text{ mol} \end{array} \right\}$	101.97	80.57	21.4	3.76
B. One-fifth complete; same as A but at one-fifth concentration.....	77.6	47.7	30.0	1.59
C. Same as B with 0.0108 mol CaCl <sub>2</sub> added.....	66.66	47.57	19.09	2.49
D. Same as B with 0.0216 mol NaNO <sub>3</sub> added.....	64.69	48.53	16.16	3.00
E. Same as B with 0.0081 mol MgCl <sub>2</sub> added.....	65.55	44.72	20.83	2.14
F. Same as B with 0.0081 mol Na <sub>2</sub> SO <sub>4</sub> added.....	72.06	47.06	25.00	1.88
G. Same as B with 0.0081 mol KCl added.....	82.88	56.76	26.17	2.16
H. Same as B with 0.0081 mol NaH <sub>2</sub> PO <sub>4</sub> added.....	48.33	33.33	15.00	2.22
I. Same as B with 0.0216 mol NaCl added.....	53.99	36.28	17.71	2.05
J. Same as A but at one-tenth concentration.....	67.97	40.27	27.77	1.45

is variation in six ions, a variation in a cation always being accompanied by an equal variation in a corresponding anion. The accomplishment of this involved the use of two triangle systems.

In one triangle (fig. 1) a series of nutrient solutions in which the anions varied were made up using three stock solutions. Solution A consisted of 0.0045 mol KNO<sub>3</sub>, 0.0045 mol Mg(NO<sub>3</sub>)<sub>2</sub>, and 0.0060 mol Ca(NO<sub>3</sub>)<sub>2</sub>. Solution B consisted of 0.0045 mol K<sub>2</sub>SO<sub>4</sub>, 0.0060 mol CaSO<sub>4</sub>, and 0.0045 mol MgSO<sub>4</sub>. Solution C consisted of 0.0060 mol Ca(H<sub>2</sub>PO<sub>4</sub>)<sub>2</sub>, 0.0045 mol MgHPO<sub>4</sub>, and 0.0045 mol KH<sub>2</sub>PO<sub>4</sub>. Plants

at one apex received solution A; at another apex, solution B; and at the third apex, solution C. The solutions in the body of the triangle were made by mixing various proportions of solutions A, B, and C, as indicated in figure 1. The concentrations of cations in the three

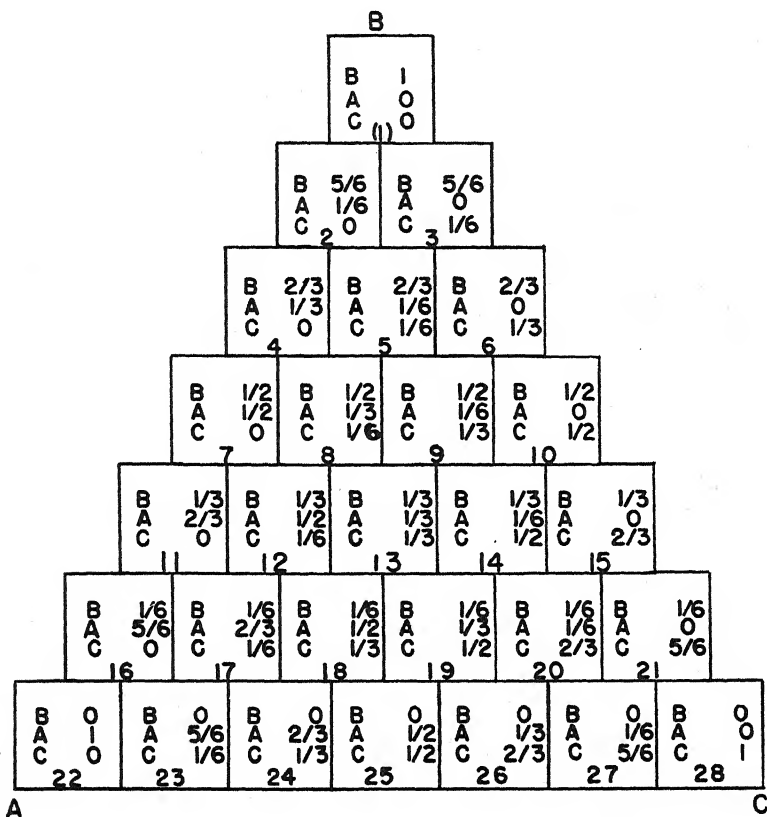


FIG. 1.—Triangle showing parts of solutions A, B, and C used in the twenty-eight different combinations. For example, square 13 contains one-third part solution B, one-third part solution A, and one-third part solution C.

solutions are practically constant, therefore these concentrations did not vary appreciably as the result of any mixture of the three solutions. In a similar manner a series of nutrients were made up in another triangle system, but in this case the cations varied and the anions remained constant. Solution D consisted of 0.0045 mol

$\text{Mg}(\text{NO}_3)_2$ , 0.0045 mol  $\text{MgSO}_4$ , and 0.0045 mol  $\text{MgHPO}_4$ . Solution E consisted of 0.0045 mol  $\text{KNO}_3$ , 0.0045 mol  $\text{K}_2\text{SO}_4$ , and 0.0045 mol  $\text{KH}_2\text{PO}_4$ . Solution F consisted of 0.0060 mol  $\text{Ca}(\text{NO}_3)_2$ , 0.0060 mol  $\text{CaSO}_4$ , and 0.0060 mol  $\text{Ca}(\text{H}_2\text{PO}_4)_2$ . These solutions were utilized and mixed in a manner similar to that described for the first triangle, except that solutions D, E, and F were used in place of solutions A, B, and C respectively (fig. 1).

Biloxi soybeans were used as the final experimental material. The seeds were selected for uniformity, transplanted after germination, and watered, all as in the previous experiments. The pots were placed in the greenhouse at  $75^\circ$ – $80^\circ$  F. The relative humidity was generally above 60 per cent.

Three plants were placed in an  $8 \times 4$ -inch glazed jar containing quartz sand. Each set in the twenty-eight squares of the triangle contained ten jars for a total of thirty plants to each square. The sand was flushed with the various nutrient solutions daily and twice a week the sand was also flushed with distilled water. The plants in experiment I were planted October 1 and harvested October 21. The plants in experiment II were planted October 7 and harvested October 26.

At harvest the roots were washed free from sand and dipped into a saturated solution of sodium chloride to remove any particles adhering, then again washed and the plant divided into two fractions, (a) the tops, and (b) the roots and hypocotyls. Fresh weights were taken and the fractions were placed in a well ventilated drying oven at  $80^\circ$  C. for two days. Dry weights were then taken by means of a torsion balance sensitive to 0.01 gm. The data were recorded on a basis of 100 plants (figs. 2, 3).

## Results

### EXPERIMENT I

In this experiment the cations were constant throughout the series of solutions and the concentration of anions varied. Plants growing in the solution containing  $\text{PO}_4$  as the only anion present (fig. 1, square 28) showed poor growth and evidences that the solution was toxic. The seedlings elongated at about the same rate as those in the other solutions, the cotyledons and first foliage leaves expanding





maximum amount of  $\text{PO}_4$  (fig. 1, square 9) the symptoms developed much the same as just described, except that new growth continued to display a few discolored areas even up to the time of harvest. Thus there is a relationship between the relative concentration of nitrate and phosphate in the nutrient and the development of phosphate toxicity symptoms, a small amount of nitrate seemingly serving in some measure to counteract what otherwise might be a toxic concentration of phosphorus. The relationship between various concentrations of phosphate and nitrate ion in the accumulation of dry weight by the plants is shown in figure 2. In general, at any particular concentration of phosphorus, more dry weight was produced with increasing concentrations of nitrate.

When nitrate only was the major anion present (fig. 1, square 22), a rate and type of growth took place which compared favorably with the greatest growth in any of the other solutions, although the total dry weight was slightly lower than in a few of the other solutions (fig. 2, squares 7, 12, 18, 26). In no case did distinct symptoms of phosphorus or sulphur deficiency appear. This may have been because during germination and early growth, such as is represented by the period of these experiments, adequate supplies of these elements were available from storage supplies in the seed. It is also possible that, even though these elements were not in the nutrient solutions supplied to certain plants, these plants may have obtained some of them from the traces present in the sand but which was free from nitrogen and phosphorus. (The seedlings were germinated in sand and watered with tap water during the early stages of germination before transplanting.) There were no evidences of sulphate toxicity or deficiency at any time.

As the amount of  $\text{PO}_4$  increased in the solution the importance of  $\text{NO}_3$  for vegetative growth and accumulation of dry weight became much more evident (fig. 2).  $\text{PO}_4$  may constitute as much as two-thirds the concentration of the anions present and yet active growth result if  $\text{NO}_3$  is present as a third part (fig. 2, square 26).

## EXPERIMENT II

Throughout the series in this experiment the anions were present at nearly the same concentration and the cations varied in relation to one another. At the time of harvest the plants showed a great

many differences between the various lots. Certain distinct characteristics varied in intensity from lot to lot. Thus some showed characteristic reddening of the petioles and midribs of the leaves, others reddish brown spots in the blades. While both these symptoms were apparent in some lots, others showed one and not the other. Because of this, certain symptoms which were characteristic of more than one lot were selected and their presence or absence recorded for each group. These patterns of response or symptoms are as follows, with the symbols used to designate them.

o Presence of red coloration:

o In petiole of primary leaves

oo In petiole of primary leaves and upper leaves

ooo In petiole and midrib of primary leaves and upper leaves

oooo In petiole and midrib of primary leaves and upper leaves and in the stem

# Presence of reddish brown spots in blade of leaf (no relative degrees of this were noted; it was either present or absent)

= Presence of light brown areas in blade of leaf; these differed from the preceding in that the color was lighter and more widespread in the leaves

g Chlorosis:

g Partial chlorosis of some leaves

gg Chlorosis general

ggg Chlorosis more general

gggg Chlorosis extreme

The presence of these symptoms and the degree to which they were manifested are shown in figure 4. A comparison of the results indicates that the reddish coloration of petioles and midribs (symptom o) represents an effect of Ca toxicity, since it is present in all plants which receive relatively high concentrations of Ca, regardless of the relative amounts of the other ions. The reddish brown spots in the blades (symptom #) represent evidence of Mg deficiency, since the symptom is present in all plants growing in magnesium-deficient solutions. The presence of light brown areas in the leaves (symptom =) represents evidence of calcium deficiency, since it is shown by all plants which grew in solutions deficient in calcium. It



of development at the time of taking samples for analysis. The present investigation has further demonstrated that such a relation between nitrogen and phosphorus in the nutrient solution also affects the rate of growth of the plants and the appearance or lack of appearance of certain toxic symptoms. LEMMERMAN and BEHRENS (6) obtained satisfactory growth of oats in pots when the proportion between  $P_2O_5$  and nitrogen was 1:1 but not when it was 3:1, in which case there was phosphate poisoning. DANIEL (3) found the nitrogen and phosphorus ratios within the plant tissue closely correlated. HUTCHINGS (5) found a close relationship in soybean between the phosphorus and nitrogen content of the plant material. BREAZEAL (1) stated that the absorption of nitrogen by wheat seedlings was not affected by the presence of either phosphorus or potassium; absorption of phosphorus was slightly increased by the presence of nitrogen. MOORE (7) found that the phosphate ion ordinarily used in nutrient solutions was injurious to peanut seedlings when grown in the light, but those grown in darkness were not subject to phosphate toxicity.

The relationship between phosphorus and nitrogen in soybean is important in overcoming toxicity of phosphorus. Phosphorus is toxic in very small concentrations if nitrogen is lacking, but it is not toxic even in fairly high concentrations if larger amounts of nitrogen are present. In general, one part of  $NO_3$  will overcome the toxic effects of two parts of  $PO_4$ . In older plants this ratio may not be the same, seedlings being more susceptible to phosphate poisoning than older plants. This is possibly caused by the greater absorption of phosphorus at the seedling stage, the lower tolerance for phosphates, or the amount of phosphorus stored in the seeds. It is possible that the older plants have enough stored nitrogen to tolerate an outside ratio which under other circumstances would be toxic.

The writer wishes to express his appreciation of the helpful suggestions given by Dr. K. C. HAMNER during the course of these experiments.

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# AVENA COLEOPTILE ASSAY OF ETHER EXTRACTS OF NODULES AND ROOTS OF BEAN, SOYBEAN, AND PEA<sup>1</sup>

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 511

GEORGE K. K. LINK AND VIRGINIA EGGERS

## Introduction

The data and conclusions reported here have resulted from attempts at preliminary coleoptile assays of ether extracts of nodules and roots of red kidney bean (*Phaseolus vulgaris*), Biloxi soybean (*Soja max*), and Alaska pea (*Pisum sativum*). The study proceeded from the hypothesis that if growth substances, that is, auxones,<sup>2</sup> play the roles in growth indicated by current experiments and theory, then the auxone relations in galls will be anomalous in one or more respects as to kind, quantity, behavior, and other relations of the relevant auxones. It seemed most feasible to begin by limiting the inquiry to the auxin<sup>3</sup> fraction of root galls.

MOLLIARD (6) reported in 1912 that sterile filtrates of cultures of *Rhizobium radiculicola* produced anomalous growth in roots of legumes. THIMANN (8) in 1936 reported that more auxin was diffused into agar from the young nodules than from the root tips of the garden pea, and that auxin applied to lateral root tips of the pea induced tumorous growth and inhibited elongation. He developed a theory to account for nodule formation in terms of the effects of auxin produced by the nodule bacteria on the cells of the invaded plant.

LINK, WILCOX, and LINK (5) in 1937, not aware of THIMANN's paper, reported that strains of *Rhizobium phaseoli* in culture pro-

<sup>1</sup> This work was aided in part by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

<sup>2</sup> The concept auxone (5) includes all growth substances, that is, all active substances which effect or affect growth in the sense of increase in size, number, and morphologic difference of wholes and of their parts. For example, it includes auxins and auxin inhibitors, thiamine, and wound hormones. It is not a chemical, but a biologic concept.

<sup>3</sup> Auxin is here used to designate that class of auxones which are readily detectable by cell elongation of the *Avena* coleoptile.

duced auxins of the indoleacetic acid type, and that this acid, applied to roots of red kidney bean, produced bending, inhibition of elongation, and tumors, depending upon the concentration of the acid, the site of application to the root, and the age of the site. A theory was advanced which took note of the possibility that in gall growth the disturbed auxone relations (dysauxony) within the host probably involve: (a) auxones contributed by the foreign agent (heteroauxones); and (b) disturbances in the normal amounts, kinds, and behavior of auxones produced by the cells of the affected plant (autoauxones). LINK (4) reported that extracts of nodules of red kidney bean were more active in growth tests with bean and tomato seedlings and *Avena* coleoptile than extracts of the roots; that they contained more auxin of the indoleacetic acid type; and concluded that the hyperauxony of the nodules probably was largely due to indoleacetic acid contributed by the bacteria.

CHEN (1) in 1938 reported that *Rhizobium trifolii* produced indoleacetic acid in culture and that the most active cultures produced the greatest quantities. GEORGI and BEGUIN (2) in 1939 reported that four species of *Rhizobium* in culture produced indoleacetic acid. In two of these, the ineffective strains (in the sense of not fixing nitrogen) produced more indoleacetic acid than the effective strains. They reported also that *B. radiobacter*, which lives in nodules as a contaminant, is able to produce indoleacetic acid. They questioned whether indoleacetic acid plays a causal role in nodule growth. THIMANN and SCHNEIDER (9) in 1939 reported that nodules of red kidney bean are at least as effective in auxin production as coleoptile tips.

### Investigation

Bean and soybean were grown in the garden in infected soil, and in the greenhouse in sterilized quartz sand watered with Shive's complete nutrient solution. The peas also were grown in the greenhouse in quartz; some inoculated, some not. The root systems were collected at different ages and at different times during the growing seasons of 1937-39. The root systems were thoroughly scrubbed and rinsed to rid them of soil and sand. The nodules were removed by hand with minimum injury to them and the roots. They were placed in ether, whole. The roots, both those from which nodules had been removed and those always free of them, were cut into pieces 1-3

inches long and then placed in ether. Preparation of ether and extraction was carried out according to VAN OVERBEEK's method (10). Extraction was continued for 22 hours. The ether extracts were evaporated to dryness and used the next or the second day in the *Avena* coleoptile test according to WENT's method (12). Each extract was taken up in 2 cc. of melted 3 per cent agar at 60° C. The

TABLE 1

ACTIVITY OF ETHER EXTRACTS OF NODULES AND ROOTS OF BEAN, SOYBEAN, AND PEA EXPRESSED IN DEGREES OF CURVATURE OF AVENA COLEOPTILES

PLANT	MATERIAL	SAMPLE (GM.)	AVERAGE CURVATURES OF TWELVE COLEOPTILES PER TEST (IN DEGREES)			
			FIRST DILUTION	SECOND DILUTION	THIRD DILUTION	FOURTH DILUTION
Bean.....	Roots (st)*	40	9.9	9.2	7.9	.....
	Roots (d-n)†	8	19.2	6.6	2.0	.....
	Nodules‡	8	18.0	19.0	23.4	21.0
Soybean.....	Roots (st)	40	12.6	12.2	8.4	.....
	Roots (d-n)	8	20.2	13.5	8.3	.....
	Nodules	8	18.7	16.2	10.3	.....
Pea.....	Roots (st)	40	19.3	21.7	9.9	.....
	Roots (d-n)	5	21.2	10.6	8.4	.....
	Nodules	5	17.6	28.3	22.1	.....
Indoleacetic acid.....			$\left\{ \begin{array}{l} 10\gamma \quad 3.7 \\ 20\gamma \quad 5.0 \\ 40\gamma \quad 12.3 \\ 60\gamma \quad 19.0 \end{array} \right\}$	Each value is average of four tests, each with twelve coleoptiles per concentration		

\* Roots (st) = roots grown in sterilized quartz.

† Roots (d-n) = roots denodulated.

‡ Nodules from denodulated sample of roots.

mixture was thoroughly stirred and then allowed to stand for an hour, after which it was remelted. A half cc. and a 0.75 cc. portion of the mixture were diluted with 0.5 cc. and 0.75 cc. of 3 per cent agar respectively. These dilutions were allowed to stand for an hour and upon remelting were poured into a mold, 8×10×1.5 mm. Each plate was cut into twelve blocks of equal volume. The blocks were applied to coleoptiles twice decapitated. For each dilution twelve test plants were used. In order that the results might be comparable for tests of different days, controls of indoleacetic acid in agar in concentrations of 10, 20, 40, and 60 gamma per liter were

run with twelve coleoptiles per test. The curvatures were recorded after 90 minutes. The data are given in table 1.

Several experiments were conducted to determine the kinds of auxins and their relative proportion in extracts of nodules and roots of the red kidney bean. To this end VAN OVERBEEK's adaptation (11) of the KÖGL, HAAGEN-SMIT, and ERXLEBEN procedure was

TABLE 2

ACTIVITY OF NON-REFLUXED AND REFLUXED ETHER EXTRACTS OF NODULES AND ROOTS OF BEAN, EXPRESSED IN DEGREES CURVATURE OF AVENA COLEOPTILES

TREATMENT	MATERIAL	SAMPLE (GM.)	AVERAGE CURVATURE OF TWELVE COLEOPTILES PER TEST (IN DEGREES)										
			FIRST DILUTION	SECOND DILUTION	THIRD DILUTION								
Not refluxed.....	Roots	8	19.2	6.6	2.0								
	Nodules	8	18.0	19.0	23.4								
H <sub>2</sub> O reflux.....	Roots	8	Sample lost	.....	.....								
	Nodules	8	15.0	19.5	20.6								
5% HCl reflux.....	Roots	8	19.6	3.6	3.6								
	Nodules	8	23.0	21.7	19.0								
0.5 N NaOH reflux.....	Roots	8	4.8	0.0	2.6								
	Nodules	8	15.0	16.0	14.5								
Indoleacetic acid.....			<table><tr><td>10γ</td><td>3.7</td></tr><tr><td>20γ</td><td>5.0</td></tr><tr><td>40γ</td><td>12.3</td></tr><tr><td>60γ</td><td>19.0</td></tr></table>	10γ	3.7	20γ	5.0	40γ	12.3	60γ	19.0	Each value is average of four tests each with twelve cole- optiles per concentration	
10γ	3.7												
20γ	5.0												
40γ	12.3												
60γ	19.0												

used. The procedure is based on the observations that: (a) indoleacetic acid is heat-stable to alkali but not to acid; (b) auxin *a* is heat-stable to acid but not to alkali; and (c) auxin *b* is heat-stable to neither acid nor alkali. Forty gm. samples of nodules and of nodule-freed roots of bean were extracted with ether for 22 hours. After extraction and partial evaporation, the ether extracts were divided each into five equal portions. One pair of these portions (one of nodule extract and one of root extract) was evaporated to dryness without further treatment; a second pair was refluxed over the steam bath for fifteen minutes with 3 cc. of H<sub>2</sub>O, adjusted as to pH (slightly

acid), taken up in ether and the ether fraction evaporated to dryness; a third pair was refluxed each with 3 cc. of 5 per cent HCl; and the fourth each with 3 cc. of 0.5 N NaOH. Each was adjusted as to pH (slightly acid), taken up in ether and the ether fraction evaporated to dryness. The residues of the eight samples were taken up in agar and tested. The data are recorded in table 2.

### Discussion

The outstanding features of the data of table 1 are: lack of linear proportionality between dilution and activity of most extracts; rise of activity values through one or several dilutions of the nodule extracts of bean and pea and of the sterile pea roots; difference in activity and dilution values of the extracts of the nodules of bean and pea compared with those of root extracts; and differences between activity values of the extracts of sterile roots and of denodulated roots.

Great care was taken to insure equal distribution of the extracts during the initial mixing with agar and in subsequent dilutions. The variations in curvature within the sets of plants tested with the first dilutions did not indicate that the lack of proportionality between dilution and activity was due to uneven distribution. It is possible that this lack of proportionality, especially of the nodule extracts, was due to supramaximal activity of the extracts in the lower dilutions. The relative magnitude of the values for the different dilutions, however, indicates that this is not an adequate explanation. It is more likely that in great part the lack of proportionality was due to change in activity of the extract during the process of mixing and dilution with agar. Phenomena noted by others and reviewed and analyzed by GOODWIN (3) and STEWART (7) possibly are responsible for the effects. The assumption that we were in part dealing with substances which do not induce or which inhibit cell elongation—but change upon hydrolysis into substances which effect cell elongation—aids a rational interpretation of the observed rise of activity of the extracts with increasing dilution. If this interpretation is correct, then the activity values recorded are not indices of auxin content of the extracts but of positive auxin balances, in the sense that the net result of the antagonistic effects of the components of the extracts on the coleoptile is cell elongation.

Since we have not yet succeeded in determining experimentally the validity of one or all of these explanations, the data are presented in their present far from satisfactory form. The order of differences recorded has appeared in many tests not included here. Because we were primarily interested in comparative values of the nodule and root extracts, these were tested first each day. As a result the indoleacetic tests, in order of decreasing concentration, came at the time of declining sensitivity of the coleoptiles. This is responsible for the disproportionately low values of the higher concentrations of indoleacetic acid relative to the lower, and to the values of the nodule and root extracts. For these reasons, in part, no attempt has been made to use the indoleacetic acid values in calculating the auxin contents of the nodules and root samples.

If the activity values of all dilutions—and especially those of the second and third—are considered, the conclusion seems warranted that either the auxin content or the positive auxin balance is greater in the nodule extracts than in the extracts of denodulated roots, and that either the auxin content or the positive balance of these, in turn, is greater than that of the extracts from roots grown in sterilized sand. The same order of differences is indicated for the extracts of the nodules, denodulated roots, and sterile roots of soybean. In the soybean the small difference between the activities of the extracts of the nodules and of the denodulated roots may be due to less complete extraction of the nodules than of the roots. Relative to the bean and pea extracts, the difference may be due to the fact that the soybean nodules were larger, smoother, and firmer than the nodules of bean and pea. The wounds made in picking them were comparatively much smaller. Hence without grinding and repeated extraction to break possible conjugate unions between auxin, auxin precursor, and other cell constituents, extraction of the soybean nodules probably was the least complete of any extraction made. The activity of the extracts of sterile soybean roots (40 gm.) relative to that of the denodulated roots (8 gm.), like that of the bean and pea roots, is decidedly lower.

The greater increase in activities with dilution in the nodule extracts of bean and pea, compared with the denodulated root extracts, may indicate that the composition of these extracts is not the same. If we assume that the extracts contain auxin inhibitors (precursors),

then the data may indicate that the nodule extracts are richer in these than the extracts of the denodulated roots, and that the denodulated roots are richer in these than the sterile roots.

The activity of the nodule extracts of bean after refluxing with NaOH and HCl was greater than that of the denodulated root extract, and the activity of the nodule extracts after acid hydrolysis was greater than after alkali hydrolysis. In accordance with VAN OVERBEEK's interpretation (11), we inferred from such data of earlier experiments that the nodule extract and root extract contained auxin *a* and indoleacetic acid, and that the nodules contained more of each, and especially more of indoleacetic acid. In the light of STEWART's findings (7) it is not impossible that the activity noted after alkali hydrolysis is not an index of presence and quantity of indoleacetic, but in part of auxin derived by hydrolysis from an auxin inhibitor or precursor. The auxin activity noted after acid hydrolysis may indicate that the roots and nodules of the bean contain something that behaves as auxin *a* in hydrolysis.

In short, the data indicate that the nodules of bean, soybean, and pea have greater and different auxone contents than the roots which bear them, and that these in turn have greater and different auxone contents than the roots when grown in *Rhizobium*-free substrates. The findings favor the hypothesis that the auxone relations in galls are anomalous.

### Summary

1. Ether extracts of nodules of bean and pea were more active in effecting negative curvatures of coleoptiles in the *Avena* test than those of denodulated roots. These in turn were more active than those of roots grown in sterilized quartz sand.
2. The same relations, but less marked, were found for nodules and roots of soybean.
3. Negative bending activity of extracts of nodules of bean and pea and of sterile pea roots was increased by agar dilution. This may indicate a fraction in each extract which changes to auxin by hydrolysis, and that the activity noted is a measure of a positive auxin balance between fractions antagonistic in their effect on cell elongation in the coleoptile. The dilution effect was most pronounced in extracts of nodules of bean and pea.
4. Extracts of nodules and of roots of bean contained a fraction

which behaved like auxin *a* in acid hydrolysis. The nodule extract contained more of it than the root extract.

5. Extracts of nodules and of roots of bean contained a fraction which was auxinic after alkali hydrolysis. The nodule extract contained more of it than the root extract. This fraction may be composed of auxin present before hydrolysis which behaves like indoleacetic acid in alkali hydrolysis, of auxin derived from a precursor by hydrolysis, or of both.

6. It is inferred that the nodules of bean, soybean, and pea have greater and different auxone contents than the roots which bear them, and that these in turn have greater and different auxone contents than the roots when grown in sterilized substrates.

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# INTERRELATION OF LIGHT AND DARKNESS IN PHOTOPERIODIC INDUCTION

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 512

KARL C. HAMNER<sup>1</sup>

(WITH FIVE FIGURES)

## Introduction

Much of the work on photoperiodism has been based on the use of 24-hour cycles, some portion of which constituted a photoperiod and the remainder a dark period. Thus any increase in the length of the photoperiod was accompanied by a corresponding decrease in the length of the dark period, or vice versa, the total length of the cycle remaining unchanged. The resulting responses were ascribed (1) to the relative extent of the photoperiod, (2) to the relative duration of the dark period, or (3) to both of these periods in combination. There has been a tendency to adhere to one or the other of the first two suggestions more than to the third. Experiments on vernalization taken in conjunction with those on photoperiodism have also received wide attention (12).

In the present investigation the possible interrelationships of reactions which result from exposure to light and reactions which result from exposure to darkness have been emphasized. Plants were chosen which are known to be very sensitive to photoperiod and which show little modification of their photoperiodic response over relatively wide ranges of temperature. No specific investigation of plants known to be thermally limited in their photoperiodic response was undertaken. Cocklebur and Biloxi soybean are generally considered short-day plants and dill and beet are classified as long-day plants. The variety of beet used, differing from many others, is not narrowly thermally limited and behaves as an annual plant. The seed was obtained from Dr. E. Carsner.

<sup>1</sup> This work was aided in part by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

### Procedure and results

The experiments recorded here were carried out during the late spring, summer, and early fall. The procedure of experimentation, the precautions employed, and the equipment used were similar to those described previously (5).

#### WORK WITH BILOXI SOYBEAN (*Soja max*)

Seeds of Biloxi soybean were planted in  $4\frac{1}{2}$ -inch clay pots containing an open garden soil. Four seeds were planted in each pot and the pots were placed on a well-lighted bench in the greenhouse. The plants were exposed to the natural daylight prevailing and supplemented from sunset until 2:00 A.M. with artificial light from Mazda filament lamps of about 100 foot-candles' intensity at the leaf surface. The photoperiod thus varied from  $18\frac{1}{2}$  to  $20\frac{1}{2}$  hours. After the seedlings had emerged from the soil and the cotyledons of most of them were fully expanded, two were removed from each pot, an attempt being made to secure maximum uniformity. The plants were well watered and grew vigorously throughout the experimental period. At the time the third trifoliate leaf was expanding they were employed for the detailed experiments.

Since it is known that Biloxi soybean requires a succession of relatively short photoperiods each followed by a relatively long dark period for floral induction, the relationship between length of the dark period and length of the light period was studied. Much of this work involved the use of cycles other than 24 hours' duration and so artificial light as well as daylight was used. In most of the experiments a number of vegetative plants were taken from the greenhouse at 5:00 P.M. and placed in a temperature-controlled room in which was installed an Eveready Carbon arc, burning sunshine carbons (8). The arc light burned continuously and furnished a quality of light similar to natural sunlight at an intensity of about 1200 foot-candles at the leaf surface. All the plants were maintained under the arc light from 5:00 P.M. until 8:00 A.M. the next morning. Including both the daylight and the arc light, therefore, all received a photoperiod of at least 26 hours at the time the different groups were subjected to the first dark period of any particular treatment. Each group was maintained subsequently at a dark period of any desired

length by placing the plants in a darkroom where they remained for the desired length of time. They were then returned to the room lighted by the arc where they were subjected to the various photoperiods. The temperature in the lighted room varied from  $73^{\circ}$  to  $78^{\circ}$  F. and in the darkroom from  $75^{\circ}$  to  $80^{\circ}$  F. The relative humidity in the two rooms varied between 50 and 80 per cent. Biloxi soybeans were employed in these experiments.

EXPERIMENT 1.—Fifty-five pots, each containing two uniform plants, were divided into eleven equal lots at 8:00 A.M., after having received the initial period of illumination. Each lot then received a 16-hour dark period. Individual lots were next exposed to the following photoperiods: 10, 11, 12, 13, 14, 16, 18, 20, 24, 30, and 36 hours. After any given lot was exposed to this photoperiod it was returned to the darkroom where it remained for 16 hours. It was then again exposed to a photoperiod equal in duration to the one it had previously received. At the end of seven cycles of such treatment each lot was maintained in the room illuminated by the arc light until 7:00 A.M. the following morning, at which time the pots were removed to the greenhouse bench and maintained at conditions of long photoperiod. Thus each treatment was ended by a long photoperiod which varied somewhat in length for the various lots, depending upon the time of day or night the treatment terminated.

EXPERIMENT 2.—Fifty-five pots of plants, selected as before, were placed in the arc-light room from 5:00 P.M. until 8:00 A.M., at which time they were divided into eleven lots. Individual lots then received the following dark periods: 8,  $8\frac{1}{2}$ , 9,  $9\frac{1}{2}$ , 10,  $10\frac{1}{2}$ , 11,  $11\frac{1}{2}$ , 12, 14, and 16 hours. Subsequent to the dark period, each lot received a 16-hour photoperiod and then was again subjected to its respective dark period. These treatments were continued until each lot had received seven cycles of treatment, subsequent to which they were treated with a period in the arc-light room as before, and then placed under long photoperiod conditions in the greenhouse.

EXPERIMENT 3.—Seventy pots of plants, selected as before, were placed in the arc-light room from 5:00 P.M. until 8:00 A.M., and then divided into fourteen lots. Individual lots then received the following dark periods: 4, 6,  $6\frac{1}{2}$ , 7,  $7\frac{1}{2}$ , 8,  $8\frac{1}{2}$ , 9,  $9\frac{1}{2}$ , 10, 11, 12, 14, and 20 hours. Subsequent to the dark period, each lot received a 4-hour photoperiod and then was again subjected to its respective dark period.

These treatments were continued until each lot had received seven cycles of treatment, subsequent to which they were illuminated for a

TABLE 1

EFFECTIVENESS OF VARIOUS CYCLES IN INDUCING FLORAL BUD DIFFERENTIATION IN BILOXI SOYBEAN (TEN PLANTS IN EACH LOT)

SEVEN CYCLES, EACH CONSISTING OF A 16-HOUR DARK PERIOD ACCOMPANIED BY FOLLOWING PHOTOPERIODS											
	LENGTH OF PHOTOPERIOD IN HOURS										
	10	11	12	13	14	16	18	20	24	30	36
No. of plants possessing floral primordia or flowers.....	10	9	9	8	8	4	2	0	0	0	0
No. of nodes possessing floral primordia or flowers.....	18	19	17	12	13	7	2	0	0	0	0

SEVEN CYCLES, EACH CONSISTING OF A 16-HOUR PHOTOPERIOD ACCOMPANIED BY FOLLOWING DARK PERIODS											
	LENGTH OF DARK PERIOD IN HOURS										
	8	8½	9	9½	10	10½	11	11½	12	14	16
No. of plants possessing floral primordia or flowers.....	0	0	0	0	0	2	3	4	5	4	5
No. of nodes possessing floral primordia or flowers.....	0	0	0	0	0	2	5	6	7	7	8

SEVEN CYCLES, EACH CONSISTING OF A 4-HOUR PHOTOPERIOD ACCOMPANIED BY FOLLOWING DARK PERIODS														
	LENGTH OF DARK PERIOD IN HOURS													
	4	6	6½	7	7½	8	8½	9	9½	10	11	12	14	20
No. of plants possessing floral primordia or flowers.....	0	0	0	0	0	0	0	0	0	0	2	3	3	4
No. of nodes possessing floral primordia or flowers.....	0	0	0	0	0	0	0	0	0	0	3	5	4	5

period in the room with the arc light as before and then placed under conditions of long photoperiod in the greenhouse.

In each experiment, at the close of the specific treatment to which

any particular lot was exposed, the plants were allowed to develop on the greenhouse bench on a photoperiod in excess of 18 hours for 3-4 weeks. The plants were then carefully dissected and the presence or absence of flowers at each node on the main axis noted.

The results (table 1; fig. 1) indicate that, regardless of the length of the photoperiod, the plants will not flower unless they receive dark periods in excess of 10 hours. If the plants receive a succession of seven cycles they may flower or not, depending upon the conditions prevailing during the photoperiods of these cycles (see later). Under

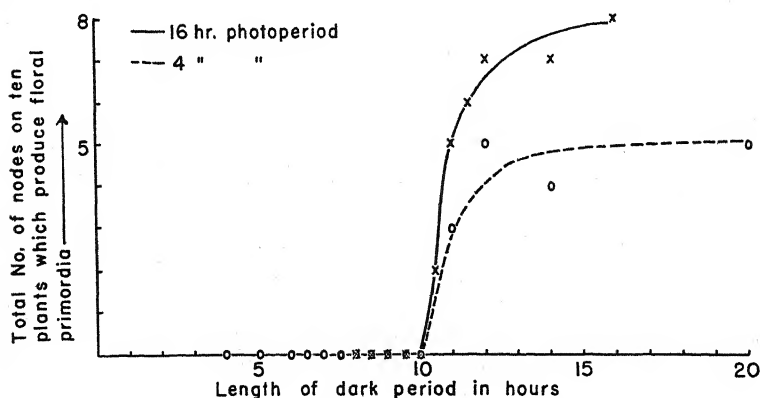


FIG. 1.—Effect of length of dark period of photoinductive cycle upon number of floral primordia produced by Biloxi soybean. All plants received seven cycles, each consisting of a 16-hour or a 4-hour light period and a dark period as indicated.

the conditions of the preceding three experiments, Biloxi soybean failed to initiate floral primordia on any cycle which did not include dark periods of more than 10 hours.

Figure 1 shows that the length of the dark period which is critical does not vary appreciably, whether the accompanying photoperiods are relatively long or short. While some floral initiation may occur as the result of exposure to a  $10\frac{1}{2}$ -hour dark period, a 12-hour dark period seems to be more effective. A 20-hour dark period, on the other hand, is neither more nor less effective than a 12-hour dark period; plants which received seven 16-hour cycles consisting of 4-hour light and 12-hour dark periods produced as many floral primordia as did plants which received seven 24-hour cycles consisting

of 4-hour light and 20-hour dark periods. This was true in spite of the fact that, at the end of the treatment, the latter plants were much smaller and the leaves were yellower than the former. Thus floral initiation may be limited by dark periods shorter than 12 hours and completely inhibited by dark periods shorter than 10 hours.

While the length of the dark period may determine qualitatively whether or not the plants will initiate floral primordia as the result of a particular treatment, the quantitative response or the number of floral primordia actually initiated is apparently determined to a

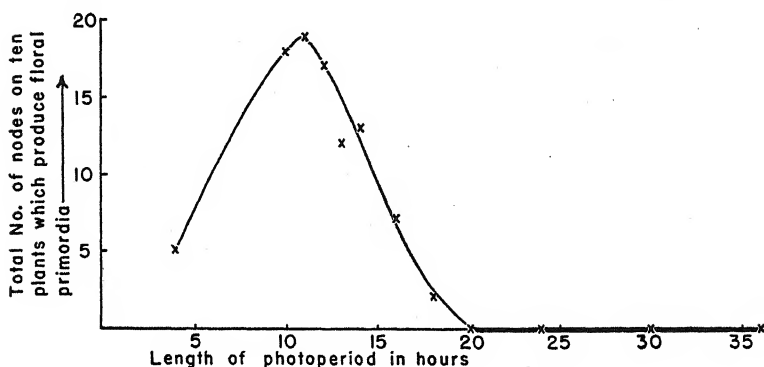


FIG. 2.—Effect of length of photoperiod upon number of floral primordia produced by Biloxi soybean. All plants received seven cycles, each consisting of a 16-hour dark period and a period of light as indicated.

marked degree by the duration of the photoperiod (fig. 2). The optimum length of photoperiod seems to be between 10 and 12 hours at a given intensity and temperature (1200 foot-candles and 73°–78° F.), and photoperiods either longer or shorter than this optimum result in the differentiation of a decreased number of floral primordia. The maximum photoperiod favorable for the initiation of floral primordia in any of the cycles used was between 18 and 20 hours and the minimum was below 4 hours.

Since these results were obtained with plants exposed to artificially controlled cycles of light and darkness, experiments were designed to compare the results with the responses of Biloxi soybean plants exposed in various ways to differing photoperiods in the greenhouse under natural sunlight.

EXPERIMENT 4.—Thirty-five pots of plants were placed on a truck which remained in the greenhouse under bright sunlight for 9 hours

TABLE 2

RESPONSES OF BILOXI SOYBEAN TO VARIOUS CYCLES, EACH CONSISTING OF 9 HOURS OF NATURAL LIGHT ACCOMPANIED BY 15 HOURS OF DARKNESS (TEN PLANTS IN EACH LOT)

	No. OF CYCLES OF TREATMENT						
	1	2	3	4	5	6	7
No. of plants possessing floral primordia or flowers.....	0	5	10	10	10	10	10
No. of nodes possessing floral primordia or flowers.....	0	7	19	24	37	47	56

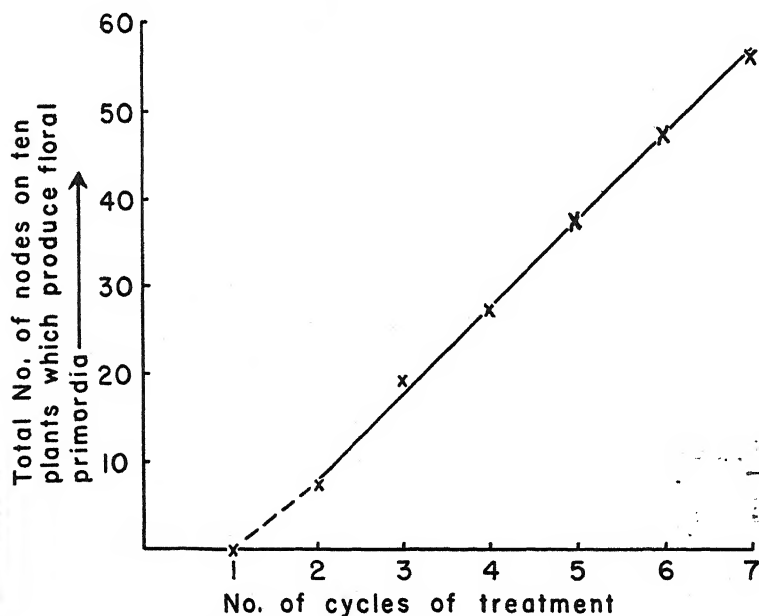


FIG. 3.—Effect of number of cycles, each consisting of a 9-hour photoperiod and a 15-hour dark period, on number of floral primordia produced by Biloxi soybean.

each day and in complete darkness for 15 hours each day. Five pots (ten plants) were removed from the truck after one such cycle of short photoperiod and long dark period, five more after two, and so

on up to seven cycles until all the plants had been removed. After treatment, the plants were returned to the greenhouse bench under long photoperiod and allowed to develop until harvested 4 weeks later. This experiment was carried out concurrently with experiments 1-3 (table 2; fig. 3).

EXPERIMENT 5.—Seventy pots of plants were placed on seven trucks, ten pots per truck, and the trucks placed in bright sunlight for 9 hours each day and then taken into separate light-tight compartments. In each compartment were suspended Mazda filament lamps controlled by time clocks, so that in addition to the 9 hours of natural light the plants on each truck could be exposed to supplementary illumination of any desired duration. Separate lots of twenty plants were each exposed repeatedly to cycles consisting of the following hours of light and dark:

Hours of light	Hours of dark	Hours of light	Hours of dark
I3.....	II	I5½.....	8½
I4.....	IO	I6.....	8
I4½.....	9½	I6½.....	7½
I5.....	9		

The results of this experiment are recorded in table 3.

TABLE 3

RESPONSE OF BILOXI SOYBEAN TO THIRTY CYCLES, EACH  
CONSISTING OF A PHOTOPERIOD AND A DARK PERIOD AS  
INDICATED (TWENTY PLANTS IN EACH LOT)

CHARACTER OF CYCLE	NO. OF PLANTS PRODUCING FLORAL PRIMORDIA OR FLOWERS
16½-hour photoperiod and 7½-hour dark period.....	0
16-hour photoperiod and 8-hour dark period.....	0
15½-hour photoperiod and 8½-hour dark period.....	0
15-hour photoperiod and 9-hour dark period.....	0
14½-hour photoperiod and 9½-hour dark period.....	0
14-hour photoperiod and 10-hour dark period.....	14
13-hour photoperiod and 11-hour dark period.....	20

Under greenhouse conditions, with bright sunlight during a portion of the photoperiod, the plants responded more rapidly than under the artificially controlled conditions of those previously discussed. Thus the ten plants which received seven cycles each consisting of 9 hours of natural light and 15 hours of darkness produced floral primordia at a total of fifty-six nodes as compared with those

plants under artificially controlled cycles of 10 hours of light and 16 hours of dark, which had floral primordia or flowers at a total of only eighteen nodes. While the total length of the artificially controlled cycle was 2 hours longer than the natural cycle, comparison seems justifiable.

The greater effectiveness of the photoperiodic treatments in the greenhouse in bringing about initiation of a greater number of floral primordia may have been associated with the fact that the temperatures there were uniformly higher than in the controlled environment; they may have been associated with the fact that the plants in the greenhouse were exposed to much higher intensities of light during their photoperiods; or there may have been other factors. It seemed most likely that light intensity was the most important factor, and a series of experiments were carried out in relation to the effect of duration and intensity of light during a short photoperiod.

EXPERIMENT 6.—One hundred and twenty vegetative plants (sixty pots) were removed from the greenhouse at 5:00 P.M. and divided into two groups of thirty pots each. One group was placed in a large darkroom and the plants arranged in six lots, each plant of any particular lot at the same distance from a 1000-watt Mazda lamp. The distances of the various lots from the lamp were such that, when the lamp was burning, respective lots received 50, 100, 150, 200, 400, and 800 foot-candles of illumination. The other group of thirty pots was placed in an adjoining darkroom so connected with the other that essentially the same temperature prevailed in both rooms at all times. The plants in this room were divided into lots and arranged in relation to a Mazda lamp as were the plants of the first room. In one room the lamp burned for 5 hours out of each 24 and in the other room for 10 hours out of each 24. The treatment was continued for 7 days, or until all plants had undergone seven cycles in the experimental room. The temperature in the experimental rooms varied somewhat, ranging from 75° to 100° F., usually being slightly higher during the photoperiod than during the dark period. After treatment, the plants were returned to the greenhouse and placed under long photoperiod, where they remained for 4 weeks, at which time they were dissected and the presence or absence of floral buds at each node noted (table 4; fig. 4).

Whether the plants were exposed to 5-hour or 10-hour photoperiods, the number of nodes possessing floral primordia or flowers

TABLE 4  
EFFECT OF DURATION AND INTENSITY OF LIGHT DURING SEVEN  
CYCLES OF SHORT PHOTOPERIOD ON INITIATION OF  
FLOWERS IN BILOXI SOYBEAN

LIGHT IN- TENSITY DURING EACH OF SEVEN CYCLES (FOOT- CANDLES)	NO. OF PLANTS IN EACH TREATMENT	NO. OF PLANTS WITH FLOWER BUDS IN THE VARIOUS TREATMENTS		NO. OF NODES WITH FLOWER BUDS ON TOTAL OF TEN PLANTS	
		5-HOUR PHOTOPERIOD	10-HOUR PHOTOPERIOD	5-HOUR PHOTOPERIOD	10-HOUR PHOTOPERIOD
50.....	10	0	0	0	0
100.....	10	0	3	0	3
150.....	10	2	10	4	14
200.....	10	4	10	6	19
400.....	10	10	10	13	29
800.....	10	10	10	31	37

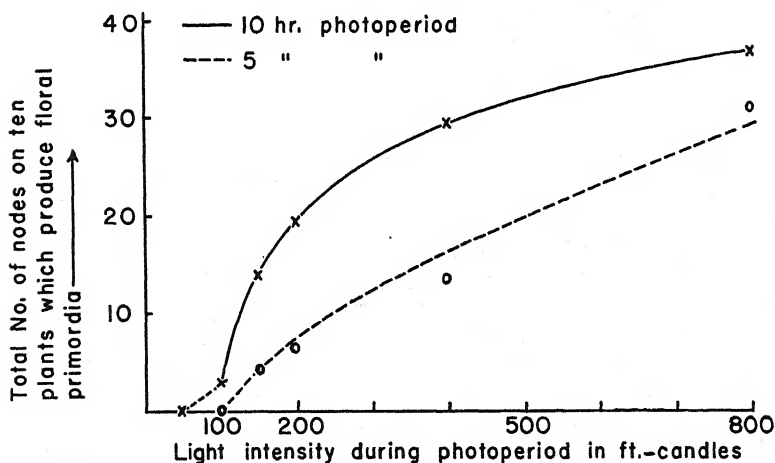


FIG. 4.—Effect of duration and intensity of light during seven photoinductive cycles on subsequent initiation of floral primordia by Biloxi soybean.

increased in proportion to the intensity of light during the photoperiod. The intensities used throughout these experiments were relatively low, and in the case of the lowest intensity used, 50 foot-candles, no flowers were produced at either photoperiod. The dura-

tion and intensity of light during the photoperiod produce a marked effect upon the number of floral primordia formed. Thus at any given intensity the number of floral primordia formed on the plant exposed to cycles with 5-hour photoperiods was approximately one-half the number produced at a comparable intensity on plants exposed to cycles with 10-hour photoperiods. Those plants which received 10-hour photoperiods developed more floral primordia at the higher intensities of light than did the plants which received a 5-hour photoperiod. From the results it would appear that any given intensity during a 5-hour photoperiod brought about a response in the plants comparable with the response brought about by one-half that intensity during a 10-hour photoperiod. It appears probable that under these conditions of relatively short photoperiod, low light intensity, long dark period, and relatively high temperature, floral initiation is roughly proportional to duration and intensity of light.

#### WORK ON THE COCKLEBUR (*Xanthium pennsylvanicum*)

Experiments with Biloxi soybean have shown that photoperiodic induction is brought about by reactions taking place during exposure to light operating in conjunction with reactions taking place during exposure to darkness. Under certain conditions the number of flowers produced may be roughly proportional to the intensity and duration of the light to which the plants are exposed. Under a variety of conditions, *Xanthium* may be induced to flower as the result of an exposure to one long dark period (5). Thus it is difficult to differentiate between the effects on floral initiation of previous photoperiods to which *Xanthium* plants have been exposed in bringing them to an age suitable for experimentation and the effects of the experimental treatment which subsequently result in their flowering.

It has been found repeatedly that *Xanthium* will not initiate floral primordia on any cycle in which the dark period is not at least more than  $8\frac{1}{2}$ –9 hours. Preliminary experiments with repeated exposures to cycles which consisted of as little as 3 minutes of light followed by 3 hours of darkness indicated that a condition might be reached such that the plants would not flower, even if subsequently exposed to a long dark period. To test out this possibility, a series of experiments were conducted.

EXPERIMENT 7.—One hundred thirty pots of vegetative *Xanthium* plants which had been growing on long photoperiod in the greenhouse for 4 weeks were removed from the greenhouse at 5:00 P.M. after a bright warm day and placed in a darkroom. Ten of the plants, serving as initial controls, were placed in a darkroom where they received no light whatsoever for 12 hours. The remaining 120 plants were placed in another darkroom in which were suspended Mazda lamps controlled by time clocks. Every 3 hours the plants in this room were exposed to light from these lamps for 3 minutes. The light intensity during this exposure was about 200 foot-candles at the leaf surface and the room temperature varied from 76° to 83° F. The

TABLE 5

EFFECT OF NUMBER OF CYCLES, EACH CONSISTING OF 3 MINUTES OF LIGHT AND 3 HOURS OF DARKNESS, ON FLORAL INITIATION IN *XANTHIUM* WHEN ALL PLANTS IMMEDIATELY SUBSEQUENT TO TREATMENT WITH SHORT CYCLES RECEIVED 12 HOURS OF COMPLETE DARKNESS (TEN PLANTS IN EACH LOT)

	No. of CYCLES OF TREATMENT												
	0	1	2	3	4	5	6	7	8	9	10	11	12
Flowering condition after 3 weeks on long photoperiod in greenhouse.....	10 fl. pr.*	6 fl. pr., 4 infl. pr.	4 fl. pr., 6 veg.	veg.	veg.	veg.	veg.	veg.	veg.	veg.	veg.	veg.	veg.

\* In this and subsequent tables dealing with *Xanthium*, the condition of the terminal bud at time of dissection is indicated as follows:

veg.: strictly vegetative  
inf. pr.: inflorescence primordia

fl. pr.: flower primordia  
mac. fl. & fr.: macroscopic flowers and fruits

plants were thus exposed to very short cycles consisting of 3 hours of darkness and 3 minutes of light. At the end of each of the twelve cycles of treatment in this room, ten plants were removed. The treatments continued for 36 hours, at which time the last ten plants were removed. Upon removal from the room, each lot of plants was exposed to 12 hours of continuous darkness in another darkroom, following which they were returned to long photoperiod in the greenhouse; or, if the treatment terminated at night, they were placed under the arc light in the room previously discussed under the experiments on soybean, where they remained until 7:00 A.M. the next morning, at which time they were removed to the greenhouse bench

alongside the other plants and continued on the conditions of long photoperiod.

All plants remained in the greenhouse for 3 weeks, after which they were carefully dissected and the presence or absence of floral buds noted (table 5).

*Xanthium* plants which have been exposed to natural light on a bright day in the greenhouse will be photoperiodically induced if they are removed at 5:00 P.M. and immediately exposed to a 12-hour dark period. On the other hand, comparable plants will not be photoperiodically induced if the exposure to the 12-hour dark period is preceded by exposure to four or more cycles each consisting of 3 minutes of light and 3 hours of darkness. These results indicate that photoperiodic induction in *Xanthium* results not only from the exposure to a long dark period but is also affected by the nature of the photoperiod which precedes it.

EXPERIMENT 8.—Since from these experiments the character of a period of light as well as a dark period seemed to influence photoperiodic induction in *Xanthium*, an attempt was made to determine what effect the duration and intensity of the light period which immediately precedes the long dark period would have. Eighty vegetative plants were removed from the greenhouse at 5:00 P.M. and treated with twelve consecutive cycles consisting of 3 hours of darkness and 3 minutes of light. The plants were then divided into eight lots of ten plants each. One lot, to serve as controls, was immediately exposed to a 12-hour dark period and then placed in the room illuminated by the arc light until the next morning, when the plants were placed and maintained on long photoperiod in the greenhouse. Five other lots were placed in bright sunlight in the greenhouse, one lot continuing there for 2 hours, one for 4, one for 6, one for 8, and one for 10. Subsequent to their respective photoperiods in the greenhouse each of the five lots was exposed to darkness for 12 hours, transferred to the arc-light room until 7:00 A.M. the following morning, and then returned to conditions of long photoperiod in the greenhouse. The remaining two lots were placed in a room at a temperature of approximately 80° F., where they were exposed to light from a Mazda lamp for 10 hours. One lot received 10 foot-candles of light and another received 100 foot-candles at the leaf surface. Subsequent to this 10-hour photoperiod each of these two lots was ex-

posed to a 12-hour dark period, transferred to the arc-light room until 7:00 A.M. the next morning, and then returned to conditions of long photoperiod in the greenhouse. The plants of all the experiments remained in the greenhouse for 3 weeks, where they received a photoperiod of 18-20 hours and a dark period of 4-6 hours during each 24-hour day. At the end of 3 weeks all plants were carefully dissected (table 6).

From the results of these two experiments it seems obvious that there is an interaction or interrelation between light and darkness in bringing about photoperiodic induction in *Xanthium*, an interrela-

TABLE 6

EFFECT OF VARYING LENGTHS AND INTENSITIES OF PHOTOPERIOD ON FLORAL INITIATION OF XANTHIUM PLANTS PREVIOUSLY CONDITIONED BY TREATMENT WITH TWELVE CYCLES, EACH CONSISTING OF 3-MINUTE LIGHT PERIOD AND 3-HOUR DARK PERIOD (TEN PLANTS IN EACH TREATMENT)

FOLLOWING PHOTOPERIODS AS INDICATED, ALL LOTS SUBJECTED TO 12-HOUR DARK PERIOD AND THEN TO LONG DAY IN GREENHOUSE	FLOWERING CONDITION* AFTER 3 WEEKS UNDER LONG DAY IN GREENHOUSE
2 hours of bright sunlight.....	5 infl. pr., 3 fl., pr., 2 veg.
4 hours of bright sunlight.....	10 fl. pr.
6 hours of bright sunlight.....	10 fl. pr.
8 hours of bright sunlight.....	5 fl. pr., 5 mac. fl. & fr.
10 hours of bright sunlight.....	4 fl. pr., 6 mac. fl. & fr.
10 hours Mazda light at 10 foot-candles.....	all veg.
10 hours Mazda light at 100 foot-candles.....	7 fl. pr., 3 infl. pr.
Controls.....	10 veg.

\* See footnote, table 5.

tionship which seems somewhat similar to the one found in soybean. A long dark period (12 hours) may or may not result in photoperiodic induction, depending upon the duration and intensity of the previous photoperiod. Plants which have been exposed to twelve cycles, each consisting of 3 minutes of light and 3 hours of darkness, are not photoperiodically induced when exposed to a long dark period of 12 hours. They are not induced if the 12-hour dark period is preceded by exposure to illumination of 10 foot-candles from a Mazda lamp for 10 hours, but they are induced if the illumination is 100 foot-candles for this same period. Thus the long dark period must be preceded by a photoperiod of more than 10 foot-candles' intensity if induction is to result. Not only is the intensity but also the duration of the light important during this photoperiod which precedes the

long dark period. An intensity of 100 foot-candles for 10 hours was more effective than bright sunlight for 2 hours, but not nearly so effective as bright sunlight for 10 hours.

While these experiments indicated that the photoperiod which precedes the long dark period plays an important part in determining whether or not exposure to a long dark period will result in initiation of floral primordia, it still remained to be determined just what part, if any, was played by exposure to light just subsequent to the dark period. In both the preceding experiments the long dark period was terminated by exposure to a long photoperiod of bright light (1200 foot-candles or more), since the plants were removed from the dark-room and immediately placed under high intensity of illumination from an arc light or in bright sunlight in the greenhouse. In another series of experiments plants were exposed to a photoperiod in the greenhouse, to a 12-hour dark period, and then immediately to varying types of photoperiod.

EXPERIMENT 9.—One hundred and thirty pots of plants were removed from the greenhouse at 5:00 P.M. and placed in the dark-room for 12 hours. Subsequent to this long dark period, during which photoperiodic induction presumably took place, the plants were divided into various lots, some lots subsequently receiving continuous illumination, some continuing in complete darkness, some receiving photoperiods of low intensity, and so on (table 7; fig. 5).

Floral initiation took place on some plants of each lot, following exposure to one 12-hour dark period, whether the plants were subsequently placed under continuous light or continuous darkness, although only a few plants initiated floral primordia during a period of 2 weeks of total darkness while every plant initiated floral primordia if returned to continuous photoperiod of high intensity in the greenhouse. Of the plants placed on continuous light of 40 foot-candles subsequent to the induction period, three out of twelve remained vegetative, and of the nine that showed floral initiation five were still in the "inflorescence primordia" stage after 3 weeks. Thus high light intensity, following the induction period, stimulates the initiation and development of flowers. The fact that plants may initiate floral primordia while in darkness indicates that, for induction, the photoperiod which ordinarily follows such an induction period apparently

is not always absolutely necessary. The results do show, however, that exposure to light, even though it is only of 40 foot-candles' intensity, does exhibit some stimulative effect upon the development of floral primordia after a long dark period of a photoinductive cycle.

TABLE 7  
EFFECT OF PHOTOPERIODIC TREATMENT SUBSEQUENT TO SINGLE  
INDUCTION PERIOD OF 12 HOURS OF DARKNESS ON  
FLORAL INITIATION IN XANTHIUM

PLANTS REMOVED FROM GREENHOUSE AT 5 P.M. AND EXPOSED TO 12-HOUR DARK PERIOD, IMMEDIATELY FOLLOWED BY TREATMENTS INDICATED	NO. OF PLANTS PER TREATMENT	FLOWERING CONDITION* AT END OF TREATMENT
Continuous light in greenhouse for 3 weeks.....	10	10 fl. pr.
Continuous darkness for 2½ weeks.....	16	5 infl. pr., 11 veg. or dead
Long photoperiod in greenhouse for 3 weeks.....	10	10 fl. pr.
Continuous light of 40 foot-candles for 3 weeks.....	12	4 fl. pr., 5 infl. pr., 3 veg.
16 hours arc light, then 3 weeks at 40 foot-candles...	8	8 fl. pr.
PLANTS PLACED ON LONG PHOTOPERIOD IN GREENHOUSE FOR 3 WEEKS SUBSEQUENT TO TREATMENTS INDICATED		
Five cycles, each consisting of 12-hour photoperiods and 12 hours of darkness.....	11	11 mac. fl. & fr.
24-hour photoperiod of 40 foot-candles.....	10	4 fl. pr., 4 infl. pr., 2 veg.
3 minutes of 5000 foot-candles, 6 hours dark, then 24 hours of 1200 foot-candles.....	12	10 fl. pr., 2 infl. pr.
30 minutes of 1200 foot-candles, 6 hours dark, then 24 hours of 1200 foot-candles.....	11	10 fl. pr., 1 infl. pr.
4 hours of 1200 foot-candles, 6 hours dark, then 24 hours of 1200 foot-candles.....	10	8 fl. pr., 2 infl. pr.
8 hours of 1200 foot-candles, 6 hours dark, then 24 hours of 1200 foot-candles.....	10	10 fl. pr.
16 hours of 1200 foot-candles, 6 hours dark, then 24 hours of 1200 foot-candles.....	10	10 fl. pr.

\* See footnote, table 5.

The foregoing experiments did not indicate the effect which light conditions following induction may have upon the continued development of floral primordia. A number of the plants exposed to continuous darkness died, the rest were very weak before completion of the experiment, and all plants at 40 foot-candles made poor growth and were yellowish in appearance while those in the greenhouse were green and thrifty. Other experiments, however (fig. 5: VII, VIII), demonstrated that it is the light condition prevailing during the first

16-24 hours following induction which largely determines the rate of development of the floral primordia. Plants which after induction were exposed to 24 hours of low light intensity (40 foot-candles) and

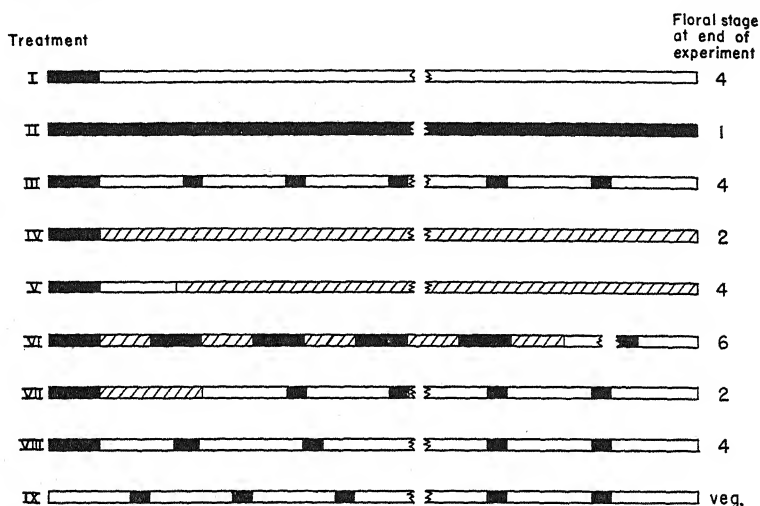


FIG. 5.—Effect of various photoperiodic treatments on *Xanthium* subsequent to photoinductive cycle. Solid black indicates periods of darkness; white, periods of light; barred sections, periods of reduced light intensity. The 12-hour phase of inductive cycle is indicated at the left for each treatment. Designation of floral stage at end of experiment is arbitrary and gives a convenient measure of relative rates of development (cf. table 7). All plants except controls (IX) were removed from the greenhouse at 5:00 P.M. after a bright warm day and exposed to 12 hours of darkness. Subsequently: I, received continuous light in greenhouse for 3 weeks; II, continuous darkness for 2½ weeks; III, cycles of 18-20 hours of light and 6-4 hours of darkness in greenhouse for 3 weeks; IV, continuous illumination at 40 foot-candles for 3 weeks; V, 16 hours illumination at 1200 foot-candles, then at 40 foot-candles for 3 weeks; VI, four and one-half cycles, each consisting of 12 hours of illumination at 40 foot-candles and 12 hours of darkness, and then 3 weeks in greenhouse on cycles with 18-20 hours of light and 6-4 hours of darkness; VII, 24-hour photoperiod at 40 foot-candles, and then 3 weeks in greenhouse as in VI; VIII, 16-hour photoperiod of 1200 foot-candles, 6 hours of darkness, 24 hours of 1200 foot-candles, and then 3 weeks in greenhouse as in VI; IX, controls, received no induction period but remained for 3 weeks under conditions of long photoperiod.

then placed on long photoperiod in the greenhouse did not develop flowers any more rapidly than did plants which received low intensity continuously. The former plants at the time of dissection were more thrifty in appearance and seemed to have abundant food

reserves, in contrast to the weak growth made by the latter. On the other hand, plants which were exposed to 16 hours of illumination from the arc light (1200 foot-candles) and then transferred to continuous low intensity (fig. 5: V) developed flowers as rapidly as did those plants which immediately after induction were placed under continuous light of high intensity (fig. 5: I). Evidently a low intensity of light following the 12-hour dark phase of the induction period does not stimulate the rate of floral development to so great an extent as does a high intensity, but it does stimulate more than does darkness. In the cases of treatment with several cycles, each consisting of 12 hours of darkness and 12 hours of low light intensity, the stimulation of floral development may be very great (fig. 5: VI).

Because of these results, and the facts that an exposure to 16 hours of bright light following the dark phase of an induction period seemed to stimulate the rate of floral development and a 6-hour dark period following such periods of bright light had little or no effect upon the rate of floral bud development (cf. fig. 5: V and VIII), experiments were devised in which the lengths of the periods of bright light were varied but each was followed immediately with a 6-hour dark period.

EXPERIMENT 10.—Two hundred vegetative plants of *Xanthium* were placed in the darkroom at 5:00 P.M. after a warm bright day in the greenhouse and exposed to 12 hours of darkness. Subsequent to this long dark period the plants were divided into ten lots of twenty plants each. One lot was continued in the darkroom for 6 more hours while another lot was placed in the room illuminated by the arc light for 12 hours and thence to conditions of long photoperiod in the greenhouse. The remaining eight lots were transferred to a room illuminated by an arc light. Here the respective lots remained for the following periods: some for 3, 10, or 30 minutes; others for 1, 2, 4, 6, or 8 hours. Subsequent to their respective exposure to bright light, all lots were transferred to a darkroom for 6 hours. At the end of this dark period they were returned to the room illuminated by the arc for at least 12 hours. When the day had become bright the following morning, all were removed to the greenhouse. After 3 weeks of the usual exposure to long photoperiod the plants were carefully dis-

sected. The various stages of development were arbitrarily classified into five groups, ranging from vegetative through four stages of inflorescence and floral development. The average stage of development was determined for each group (table 8). These results are inconclusive but indicate that a short photoperiod following the 12-hour dark phase of a photoinductive cycle of induction results in a

TABLE 8

EFFECT OF VARIOUS SHORT PHOTOPERIODS IMMEDIATELY FOLLOWING  
12-HOUR DARK PHASE OF A PHOTOINDUCTIVE CYCLE ON  
FLORAL INITIATION IN *XANTHIUM*

VEGETATIVE PLANTS REMOVED FROM GREENHOUSE AT 5:00 P.M. AND EXPOSED TO 12-HOUR DARK PERIOD, THEN TO VARIOUS CYCLES INDICATED. SUBSEQUENTLY PLACED IN ROOM ILLUMINATED BY ARC LIGHT FOR AT LEAST 12 HOURS, REMAINING THERE UNTIL THEY COULD BE TRANSFERRED TO BRIGHT SUNLIGHT IN THE GREENHOUSE THE NEXT DAY

LENGTH OF PHOTOPERIOD (1200 FOOT-CANDLES)	LENGTH OF DARK PERIOD (HOURS)	NO. OF PLANTS IN EACH TREATMENT	NO. OF PLANTS WITH FLORAL PRIMORDIA	AVERAGE STAGE OF FLORAL DE- VELOPMENT*
0 minutes.....	6	20	20	2.0
3 minutes.....	6	19	18	2.1
10 minutes.....	6	20	19	2.6
30 minutes.....	6	20	17	1.9
1 hour.....	6	20	20	2.6
2 hours.....	6	20	20	2.8
4 hours.....	6	19	19	2.8
6 hours.....	6	20	17	2.1
8 hours.....	6	20	19	2.8
12 hours.....	0	20	20	3.2

\* The various stages of development exhibited were arbitrarily classified into five groups: vegetative and four stages in inflorescence and flower development.

slower rate of development than a long one. They confirm the results recorded in table 7, that dark periods of 6 hours or less after the photoinductive cycle have no effect on floral initiation or development.

#### SO-CALLED LONG-DAY PLANTS, DILL AND BEET

As has been shown, *Xanthium* and Biloxi soybean initiate floral primordia only when exposed to photoinductive cycles made up of both light and dark periods. Dill (*Anethum graveolens* Linn.), as grown under the varying cycles of light and dark periods occurring in nature, would be classified as a long-day plant (6). Among many other experiments not cited here, however, one was carried out during

early summer in which seeds of dill were planted in pots in soil so near the surface that most of them actually were visible when viewed from above. The pots were immediately placed in the greenhouse where they received natural illumination during the day and artificial illumination of about 100 foot-candles throughout the night. The seeds, seedlings, and developing plants were thus exposed to continuous light. Within 5 weeks most of the plants were in flower, although small and weak with slender flower stalks and relatively few foliage leaves. It is obvious that this plant requires no dark period whatsoever during growth and development to differentiate floral primordia and blossom.

The beet (*Beta vulgaris*) as ordinarily grown is a biennial, flowering during the second season after planting the seeds. Seeds of an annual strain were obtained from Dr. Ewbanks Carsner, who designated it, in so far as its flowering was concerned, as a long-day plant. Many experiments have been conducted with it in the greenhouse. The following results were obtained from some experiments during spring and summer and are pertinent here.

Seeds were planted in ordinary greenhouse flats in January. These were placed on trucks in the greenhouse and exposed to cycles of 9 hours of natural daylight and 15 hours of darkness. The temperature varied during this work but was never below 60° F. After the seedlings had developed four or five foliage leaves and were of sufficient size they were transplanted singly to 3½-inch clay pots. The plants continued on this same cycle until used in the experiment, at which time they were about 6 weeks old. Only uniform plants were used in the several experiments.

In late March ninety-six uniform plants were transferred to cycles, each consisting of approximately 19 hours of light and 5 hours of darkness. Subsequently, every few days eight plants were returned to conditions in which the cycle consisted of 9 hours of light and 15 hours of darkness. This was done in order to determine the number of long photoperiods necessary for induction. The results indicated that more than fifteen long photoperiods were required, although the exact number was not determined with accuracy at this time. Later experimentation showed that the induction period lay somewhere between fifteen and twenty cycles, consisting of 19 hours of light and 5 hours of darkness. These experiments are not dis-

cussed in detail although the results from one of them, carried out at the same time as the experiments listed below, are included in the table with them for comparison.

On May 11, 620 vegetative plants growing on cycles of 9-hour light and 15-hour dark periods were selected for uniformity. Ten were continued on this cycle as controls. These remained vegetative. The remaining 610 were all exposed to ten cycles, each consisting of 18- to 20-hour light periods and 6- to 4-hour dark periods. Subsequent to this 10-day period, 600 of the plants were returned to cycles of short photoperiod, as noted above, and the remaining ten were continued on the cycles of long photoperiod. Subsequent treatment of the plants and the results obtained are given in table 9. The results were recorded 4 weeks after the last transfer was made. The date of harvesting was sufficiently late so that presumably any plants responding to the treatment would have had time to flower.

These results indicate that this particular strain of beet requires from fifteen to twenty cycles of long photoperiods for induction, whether such photoperiods are received in direct succession or received as two separate treatments with varying numbers of cycles of short photoperiod or long dark period intervening. This seems to be true even though as many as sixteen cycles of short photoperiod intervene between the two treatments with cycles of long photoperiod. When as many as thirty-two cycles of short photoperiod intervene, there are indications that a few additional cycles of long photoperiod may be necessary in order to complete the induction; but it is certain that a treatment with as many as thirty-two cycles of short photoperiod will not nullify to any appreciable extent the effect of a previous treatment with cycles of long photoperiod, even though the treatment with the latter was not sufficiently prolonged to bring about the induction of flowering or any visible change in the growth condition of the plant.

### Discussion

Beet and dill, the two long-day plants studied, differ in many respects from the two short-day plants, *Xanthium* and Biloxi soybean, so far as their flowering responses are concerned. Dill may be germinated and grown in continuous light and within a few weeks

TABLE 9

FLORAL RESPONSES OF BEET PLANTS SUBJECTED TO VARIOUS NUMBERS OF PHOTOPERIODIC TREATMENT

TYPES OF PHOTOPERIOD TREATMENT	TOTAL NO. OF CYCLES OF LONG PHOTOPERIOD TO WHICH PLANTS WERE EXPOSED, TREATMENT IN MANY CASES BEING INTERRUPTED BY CYCLES OF SHORT PHOTOPERIOD AS INDICATED IN COLUMN 1											
	14	16	18	20	22	24	26	28	30	35	40	45
A. Controls: all cycles of long photo- period given in consecutive order.....	7 veg., 3 fl.	1 veg., 8 fl.	1 veg., 8 fl.	1 veg., 8 fl.	.....	.....	.....	.....	.....	.....	.....	.....
B. Two cycles of short photoperiod between exposure to 10th and 11th cycles of long photoperiod.....	10 veg.	7 veg., 3 fl.	2 veg., 8 fl.	10 fl.	10 fl.	10 fl.	10 fl.	10 fl.	10 fl.	10 fl.	10 fl.	10 fl.
C. Four cycles of short photoperi- od between exposure to 10th and 11th cycle of long photo- period.....	10 veg.	9 veg., 1 fl.	5 veg., 5 fl.	7 fl., 3 veg.	10 fl.	9 fl., 1 dead	10 fl.	10 fl.	9 fl., 1 dead	10 fl.	10 fl.	10 fl.
D. Eight cycles of short photoperi- od between exposure to 10th and 11th cycles of long photo- period.....	10 veg.	7 veg., 3 fl.	2 veg., 8 fl.	10 fl.	9 fl., 1 dead	10 fl.	10 fl.	10 fl.	10 fl.	10 fl.	10 fl.	10 fl.
E. Sixteen cycles of short photope- riod between exposure to 10th and 11th cycles of long photo- period.....	10 veg.	5 veg., 5 fl.	2 veg., 8 fl.	10 fl.	9 fl., 1 dead	10 fl.	10 fl.	10 fl.	10 fl.	10 fl.	10 fl.	10 fl.
F. Thirty-two cycles of short pho- toperiod between exposure to 10th and 11th cycles of long photoperiod.....	10 veg.	10 veg.	9 veg., 1 fl.	7 veg., 3 fl.	3 veg., 7 fl.	1 veg., 9 fl.	10 fl.	10 fl.	10 fl.	10 fl.	10 fl.	10 fl.

develop to the flowering stage with no dark period whatsoever, a fact which is not true for either *Xanthium* or the soybean. Thus it appears that, whatever effect exposure to a dark period may have in the latter two plants, darkness is not required for photoperiodic induction in dill. It is not yet clear whether the particular variety of beet used could be induced to flower without exposure to darkness, since such experiments have not been conducted. It is clear that beet differs from Biloxi soybean and *Xanthium* in that photoperiodic induction occurs when exposed to cycles having relatively long photoperiods (18-20 hours) and short dark periods. There is also a marked cumulative effect of exposure to such cycles even though the cycles may be interrupted by those which are not favorable for induction.

Biloxi soybean and *Xanthium* appear to be similar in that, for photoperiodic induction to take place, they must be exposed to cycles of light and darkness in which the light periods are of a certain intensity and duration and the dark periods of a definitely minimum duration. Since most of the experiments recorded in this paper have dealt with Biloxi soybean and *Xanthium*, and since the few responses of beet and dill studied seem to differ in many respects from them, this discussion deals entirely with an attempt to analyze the responses of Biloxi soybean and *Xanthium*.

Since the specific length and character of both the photoperiod and the dark period determine the results of photoperiodic induction in both plants, it appears that determinative reactions take place during both phases of the cycle, and also that there is an interaction among them. For convenience and brevity of reference, the changes or conditions which arise owing to exposure to light may be designated as A, those owing to darkness as B, and the possible summation or resultant changes related to A and B may be referred to as C. Thus A, B→C.

It is assumed that through the medium of C the observable effects, such as differentiation of floral primordia, floral development, and the like are manifested. Through C, therefore, both A and B would at present have to be investigated. Experimental evidence indicates this is possible. Thus manipulations of conditions during the light period which affect the expressions of C have been

made, and are of such character that it is clear they must be related specifically only to conditions prevailing during the light period. The same is true for other experiments related to the conditions during the dark period. During periods of darkness the degree of accumulated change in A might remain the same or gradually disappear; and similarly the degree of change in B might accumulate during periods of darkness and either persist or disappear during periods of light. The effectiveness of A and B and the relative persistence of either or both during particular conditions of cyclic alternation of light and darkness might presumably determine the changes relating to and the effectiveness of C. As in the cases of A and B, C could also disappear, dependent upon the various environmental factors involved. The experiments listed in this paper were largely designed to investigate these various possibilities and are discussed in relation to them.

#### EVIDENCE RELATIVE TO A AND FACTORS INFLUENCING IT

**BILOXI SOYBEAN.**—Many suggestions have been made in attempts to explain various types of photoperiodic evidence. Some have considered the light phase as primarily important, others the dark phase, and still others various interactions of the two. BORTHWICK and PARKER (2) have shown that Biloxi soybean will initiate floral primordia as the result of exposure to three 24-hour cycles, each consisting of a short light period and a long dark period, but will not initiate floral primordia as the result of exposure to 72 hours of darkness nor as the result of exposure to continuous light. As has been shown in experiments 1, 2, and 3 and illustrated by figure 2, under the influence of cycles in which each dark period is of 16 hours' length the number of floral primordia initiated is dependent upon the duration of the light period, increasing up to exposures of 11 hours and decreasing with longer exposures. Also the number of primordia formed is dependent upon the intensity of the illumination, increasing with increasing intensities (experiment 6 and fig. 4).

**XANTHIUM.**—Previous work (5) has shown that floral initiation in *Xanthium* is ordinarily limited by reactions taking place during the dark period. The plants under 24-hour cycles of alternating light and dark periods, in which the light period exceeds 4 hours, pre-

sumably experience an increased effectiveness of A and initiate floral primordia whenever the plant is exposed to cycles having dark periods of 9 hours or more, which permits an increased effectiveness of B. In order to demonstrate the effect of conditions which influence A, it was first necessary to obtain plants at a minimum of A. The procedure actually used to secure such plants is outlined in experiments 7 and 8. After being removed from the greenhouse, the plants were not immediately exposed to a long dark period, but instead to short cycles each consisting of 3 minutes of light and 3 hours of darkness. After exposure to four such cycles (experiment 7 and table 5) the plants did not become photoperiodically induced, even though subsequently exposed to a long dark period. The possible explanation of such responses might be as follows. During such short cycles, the opportunity for an increase in the effectiveness of A is slight since the duration of each photoperiod is only 3 minutes. Not only does A not increase greatly under these conditions but it actually decreases until, after four cycles, it becomes so slight that photoperiodic induction does not occur. During exposure to such short cycles B does not increase in effectiveness because the dark periods of each cycle are too short, but there was abundant opportunity for increase of B during the 12 hours of darkness following the last cycle. The progression A, B→C did not follow because of the deficiency of A. That the deficiency of A limited the behavior is further indicated as probable by experiment 8. Plants were exposed to twelve of the short cycles. Such plants failed to initiate floral primordia if they were immediately exposed to 12 hours of darkness, but they did initiate floral primordia if, just previous to exposure to the 12-hour dark period, they were exposed to a photoperiod of 2 hours of bright sunlight or 10 hours of light from a Mazda lamp at 100 foot-candles so that there was an opportunity for an increased effectiveness of A. Under such conditions the rate of floral development was roughly proportional to the duration and intensity of the light.

Thus in *Xanthium*, as in Biloxi soybean, it appears that there are definite, modifiable conditions during exposure to light which quantitatively influence the results of the photoperiodic reaction as a whole.

## EVIDENCE RELATIVE TO B AND FACTORS INFLUENCING IT

**XANTHIUM.**—Repeated experiments with *Xanthium* (5, 7) have shown that photoperiodic induction does not take place unless the dark period of any cycle exceeds  $8\frac{1}{2}$  hours. Whatever the reactions are that take place during exposure to darkness, they seem to be inhibited by light (5, 11) even of very low intensity. A dark period of 9 hours which would be effective in floral initiation may be completely ineffective if interrupted by as little as one minute of light. Exposure of plants to low temperatures during the dark periods greatly reduces their effectiveness, a much longer time being required under such conditions for induction to take place (5, 3). These results, together with the results of experiment 7, indicate that there is an increasing effectiveness of B during exposure to darkness, and that, whatever B is, there is no response resulting in C up to a certain threshold value. At temperatures of  $65^{\circ}$ – $80^{\circ}$  F., this threshold value is passed after  $8\frac{1}{2}$ –9 hours of darkness.

The results also indicate that, up to the threshold value, whatever degree of B has been attained during exposure to darkness, it may be completely or almost completely negated by a very brief exposure to light. As already stated, one minute of light which interrupts a 9-hour dark period completely nullifies the effect of the entire period of darkness prior to the interruption, and another period of complete darkness (9 to 12 hours) is necessary to be effective (experiment 7).

**BILOXI SOYBEAN.**—That photoperiodic induction in Biloxi soybean will not take place unless the dark period is definitely more than a certain minimum duration is strikingly illustrated in figure 1 and experiments 2 and 3. BORTHWICK and PARKER have obtained with Biloxi soybean much the same results as were obtained with *Xanthium* with brief exposures to light (2) and with exposures to low temperatures during the dark period (3).

A consideration of the two plants, in so far as conditions of A and B are concerned, demonstrates their many similarities. As already suggested, it would seem likely that in both plants the A, B→C relationship might hold. The effectiveness of A accumulates during exposure to light and slowly decreases during exposure to darkness. In conjunction with B which increases during exposure to darkness

and may decrease very rapidly during exposure to light, C results, and floral initiation takes place. Many experiments show that the rate of development of flowers, fruits, and the like is influenced by environmental factors, especially the level of nutrition (4, 9, 10) subsequent to the photoinductive period.

#### EVIDENCE RELATIVE TO C AND FACTORS INFLUENCING IT

**XANTHIUM.**—The direct results, such as floral initiation, which are assumed to be specifically related to C, also show that it probably varies quantitatively and may be influenced by conditions immediately following the photoinductive period. Thus in *Xanthium* initiation of floral primordia may follow a single 24-hour cycle of 12 hours of light and 12 hours of darkness. This is true whether the plants are maintained immediately subsequently in total darkness or in continuous light (experiment 9 and fig. 5). Also, plants which have been induced may continue to flower and set fruit even though subsequently exposed for 7 months to continuous light (5, 10).

The responses to C do not seem to represent strictly an "all or none" reaction. It has been found repeatedly that while one cycle of treatment may result in floral initiation, two cycles result in more rapid development of the primordia into flowers and fruit, three cycles in still more rapid development, and so on. It seems probable therefore that the effectiveness of C is not rapidly destroyed and may be intensified, resulting in a more rapid development of primordia into flowers and fruits, depending upon nutritional and other environmental factors as well. The experiment recorded in table 7 and figure 5 could be interpreted on this basis.

**BILOXI SOYBEAN.**—The evidence in the case of Biloxi soybean relating to C is of much the same nature as in *Xanthium*, in that induction takes place only in relation to a certain number and character of photoinductive cycles (experiments 1, 2, 3, 4, and 5; figs. 1, 2, 3, and 4). While in *Xanthium* the number of photoinductive cycles to which plants were exposed affected the rate of floral development, in Biloxi soybean not only does the rate of floral development depend upon the number of cycles of treatment (1), but also the total number of floral primordia formed increases almost in direct proportion

to the number of photoinductive cycles of treatment. One cycle does not result in the initiation of any primordia (table 2; fig. 3).

In Biloxi soybean, in contrast to *Xanthium*, while C may arise in much the same manner, several photoinductive cycles are required for it to become sufficiently effective to result in the initiation of floral primordia. Evidence for such a hypothesis is available from at least two sources. First, the evidence from the present experiments as well as those of others indicates that C may be intensified through two or three consecutive photoinductive cycles before there is initiation of floral primordia. Second, the experiments of LONG (7) showed that, following three photoinductive cycles, floral initiation took place to a certain extent and then when the plants were placed under conditions of long photoperiod no more flower primordia were initiated. If another series of photoinductive cycles of short photoperiod were used on the same plants, however, then additional floral primordia would be differentiated by them, the number of primordia being in proportion to the number of such cycles employed. Thus it was possible to induce plants to flower, have them develop vegetatively for a time, and then develop primordia more or less at will; but in each instance the plants behaved as though they had not been previously induced, or as though there was no prolonged carryover of the effects of C unless the photoinductive cycles were virtually consecutive.

From these experiments it could be assumed that C increases in soybean much as it does in *Xanthium*, as the result of exposure to photoinductive cycles. Differing from *Xanthium*, however, C is insufficient in any one cycle to bring about subsequent floral initiation, or it may not persist but disappear rather rapidly during exposure to light. Such an assumption could explain the results of LONG. Unless exposure to the photoinductive cycles occurred in direct succession, there was no effectiveness of C from one cycle to another, owing to its disappearance over intervening periods, and it never passed the threshold value. In order that additional primordia might be initiated, it was necessary to subject plants to several photoinductive cycles.

Such an explanation also would cover the results illustrated in

figure 2. Cycles which include photoperiods in excess of 20 hours do not result in photoperiodic induction even though the dark periods of such cycles are 16 hours in duration. Since A increases in effectiveness during light, presumably a 20-hour photoperiod should result in an intensification of A; and since a 16-hour dark period is long enough for B to increase above its threshold value, then in a cycle consisting of a 20-hour photoperiod and a 16-hour dark period a certain effectiveness of C should result. An explanation for the failure of such cycles to result in floral initiation could be that during the long photoperiod of each cycle the conditions of C which resulted during the previous cycle disappeared. Thus, in figure 2, the downward slope of the curve of floral initiation with increasing photoperiod could represent the effect of photoperiod on the dissipation of C.

### Summary

1. Experimental work has been presented on the effect of various cycles of light and darkness on photoperiodic induction of Biloxi soybean, *Xanthium*, dill, and beet.

2. Experimental evidence has indicated that in both the soybean and *Xanthium* photoperiodic induction is dependent in part upon responses which occur as the result of exposure to light. For purposes of discussion, these responses have been referred to as A.

3. Experimental evidence has indicated that in both *Xanthium* and soybean photoperiodic induction is dependent in part also upon responses which result from exposure to darkness. For purposes of discussion, these have been referred to as B. Photoperiodic induction results only after periods of darkness which exceed a certain minimum length.

4. For convenience and brevity of reference, the changes or conditions related to exposure to light may be designated as A, those owing to darkness as B, and the resultant changes related to A and B may be referred to as C.

5. The establishment of the categories A, B→C as here suggested is an aid toward an interpretation of the behavior of *Xanthium* and Biloxi soybean. Its extension to the interpretation of the photoperiodic behavior of other plants may be possible.

6. Additional experiments, so performed that they may be subjected to such a type of analysis, are in progress.

The assistance given by A. W. NAYLOR, L. K. MANN, and W. E. SNYDER in carrying out some of these experiments is greatly appreciated.

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# EFFECT OF NAPHTHALENE ACETIC ACID AND NAPHTHALENE ACETAMIDE ON NITROGENOUS AND CARBOHYDRATE CONSTITUENTS OF BEAN PLANTS

JOHN W. MITCHELL<sup>1</sup>

(WITH TWO FIGURES)

## Introduction

The application of alpha naphthalene acetamide to the stems of young bean plants results in such characteristic histological and morphological responses as inhibited longitudinal growth of internodes without marked increase in stem thickness, and increased cambial activity in the stem near the treated region, with formation of a relatively large amount of secondary thickening (2, 4). Comparable application of alpha naphthalene acetic acid results in meristematic activity of cells of several tissues of the stems near the treated region, and finally in the initiation of numerous root primordia on the stem (1, 4). Elongation of the internodes and expansion of the leaves of the terminal buds is retarded as a result of application of naphthalene acetamide, and also naphthalene acetic acid, to the stems of bean plants. Similar responses have been observed in plants sprayed with lanolin emulsions containing these compounds (4).

In the present investigation some chemical responses that resulted from treating plants with naphthalene acetamide are compared with those that resulted when the plants were treated with naphthalene acetic acid or left untreated.

Lanolin mixtures of naphthalene acetamide and naphthalene acetic acid were applied individually to the stems of the plants. These were later analyzed and the amount, and to some extent the type, of carbohydrate and nitrogenous compounds present in several portions of the plants compared.

## Methods

Approximately 3000 kidney bean plants were grown in sand with a complete nutrient solution containing nitrogen in the form of calcium

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nitrate. The nutrient was first applied when the plants emerged from the sand; sufficient later applications were made to keep the sand at optimum moisture content. The greenhouse temperature varied between  $70^{\circ}$ – $75^{\circ}$  at night and  $75^{\circ}$ – $85^{\circ}$  F. during the daytime. The light intensity was relatively high, as clear weather prevailed during the experiment.

Two per cent lanolin mixtures of alpha naphthalene acetamide and alpha naphthalene acetic acid were applied individually as a ring 2 mm. wide around the first internodes, midway between the first and second nodes, when the plants were 10–12 cm. tall and the first internodes approximately 15 mm. long. Control plants were treated in a similar way, using pure lanolin. The treatments were distributed evenly throughout the entire number of plants grown. The plants were harvested 12 days following treatment, at which time they showed marked responses and had not yet produced flowers.

Plants were carefully removed from the sand and the roots washed and dried on paper toweling. Approximately 500 plants from each treatment were used for nitrogen determinations. These were divided into the following portions: (1) roots and hypocotyls; (2) first internodes; and (3) tops, including all leaves, internodes, and petioles above the first internode.

Samples for carbohydrate determinations were collected from 300–500 plants in each treatment and divided into five portions: roots, hypocotyls, first internodes, primary leaves, and all parts above the first internode other than primary leaves (which were designated tops).

Fresh tissue analyzed for nitrogenous compounds was ground to a fine pulp and moisture samples were taken, the solids from which were later used for total nitrogen determinations by means of the Kjeldahl method. Soluble nitrogen fractions were removed from aliquots repeatedly ground with cold water in quartz sand and filtered through cloth. Following clarification with acetic acid, ammonia and nitrate nitrogen in the extract were determined as described by SESSIONS and SHIVE (7), and the total nitrogen by the Kjeldahl method.

Sugars were extracted with 80 per cent alcohol from tissue pre-

viously dried and ground to 60 mesh. The extracts were cleared with neutral lead acetate and the non-reducing sugar hydrolyzed with invertase. Reducing power of the resulting solutions was determined by a micromethod (5) with modifications as suggested by PHILLIPS (6), the copper being dissolved in ferric alum and the iron titrated with potassium permanganate.

Starch was digested with saliva in samples ground to 100 mesh, hydrolysis was completed as described by LOOMIS and SHULL (3), and reducing power of the cleared solutions was determined by the method described.

### Experimental results

#### GROWTH RESPONSES

Two hours after treatment with naphthalene acetic acid the plants showed marked stem curvatures, which persisted throughout the experiment. Tumors developed on the first internodes near the treated region within 3-4 days after treatment. Plants treated with the acetamide mixture or pure lanolin showed no curvatures or tumor formation during the experiment. Both naphthalene acetic acid and naphthalene acetamide inhibited the expansion of internodes, leaves, and petioles. Terminal buds failed to increase appreciably in size during the 12 days following application of naphthalene acetic acid to the first internodes. At the time of harvest, first internodes treated with naphthalene acetamide and naphthalene acetic acid measured approximately 2 inches in length, while those to which pure lanolin was applied were approximately 3 inches.

Numerous roots formed on the hypocotyls and first internodes of plants treated with the acid, and a smaller number were evident only on the lower part of the hypocotyls of plants treated with the acetamide.

#### CHEMICAL RESPONSES

Naphthalene acetamide and naphthalene acetic acid, applied in lanolin to the first internodes of bean seedlings, resulted in chemical responses in the stems near the treated region, and also in all other portions of the plants that were analyzed. Considering the plant as a whole, the amount of solid matter synthesized by those treated with the acetamide and the acid was less, while the water content meas-

ured as percentage moisture in fresh tissue was greater than that of controls (tables 1, 2). All portions, except the primary leaves of

TABLE 1

WEIGHT OF FRESH AND DRY TISSUE OF BEAN PLANTS 12 DAYS FOLLOWING TREATMENT WITH 2 PER CENT LANOLIN MIXTURE OF NAPHTHALENE ACETAMIDE AND NAPHTHALENE ACETIC ACID, AS COMPARED WITH OTHERS TREATED WITH PURE LANOLIN. FIGURES REPRESENT GRAMS SOLID MATTER PER 100 PARTS

PLANT PART	LANOLIN		NAPHTHALENE ACETAMIDE		NAPHTHALENE ACETIC ACID	
	FRESH WEIGHT	DRY WEIGHT	FRESH WEIGHT	DRY WEIGHT	FRESH WEIGHT	DRY WEIGHT
Roots.....	110.8	11.3	101.8	10.7	115.1	8.6
Hypocotyls.....	90.1	11.1	90.3	9.7	101.2	9.0
First internodes.....	37.0	5.6	34.0	5.2	153.1	19.2
Primary leaves.....	304.9	34.2	307.2	33.2	244.7	29.3
Tops*.....	620.9	68.7	245.0	28.8	8.2	1.0
Entire plant.....	1163.7	130.9	808.3	87.6	622.3	67.1

\* All portion above second node.

TABLE 2

MOISTURE CONTENT OF PLANTS TREATED WITH 2 PER CENT LANOLIN MIXTURES OF NAPHTHALENE ACETAMIDE AND NAPHTHALENE ACETIC ACID AS COMPARED WITH OTHERS TREATED WITH LANOLIN

PLANT PART	LANOLIN		NAPHTHALENE ACETAMIDE		NAPHTHALENE ACETIC ACID	
	PER 100 PARTS (GM.)	PERCENTAGE FRESH WEIGHT	PER 100 PARTS (GM.)	PERCENTAGE FRESH WEIGHT	PER 100 PARTS (GM.)	PERCENTAGE FRESH WEIGHT
Roots.....	99.5	89.8	121.1	91.8	106.5	92.5
Hypocotyls.....	79.0	87.6	80.6	89.2	92.2	91.1
First internodes.....	31.4	84.8	28.8	84.7	133.9	87.4
Primary leaves.....	207.7	68.1	274.0	89.1	215.4	88.0
Tops.....	552.2	88.9	216.2	88.2	7.2	87.4
Entire plant...	969.8	83.3	720.7	89.2	555.2	89.2

plants treated with the acid, contained a lower percentage of carbohydrate in the form of starch, dextrin, and sugar than did controls (table 3). Similarly the roots, hypocotyls, and first internodes of

TABLE 3

AMOUNT OF STARCH, DEXTRIN, AND SUGAR IN PLANTS 12 DAYS FOLLOWING TREATMENT WITH 2 PER CENT MIXTURE OF LANOLIN-NAPHTHALENE ACETAMIDE AND LANOLIN-NAPHTHALENE ACETIC ACID, AS COMPARED WITH OTHERS TREATED WITH LANOLIN

PLANT PART	BASIS OF CALCULATIONS					
	PER 100 PARTS (MG.)			PERCENTAGE SOLID MATTER		
	LANOLIN	NAPH- THALENE ACETA- MIDE	NAPH- THALENE ACETIC ACID	LANOLIN	NAPH- THALENE ACETA- MIDE	NAPH- THALENE ACETIC ACID
TOTAL SUGAR						
Roots.....	215	139	112	1.9	1.3	1.3
Hypocotyls.....	599	233	198	5.4	2.4	2.2
First internodes.....	196	130	442	3.5	2.5	2.3
Primary leaves.....	1300	1162	1436	3.8	3.5	4.9
Tops.....	2473	778	25	3.6	2.7	2.5
Entire plant.....	4783	2442	2213	3.6	2.8	3.3
STARCH AND DEXTRIN						
Roots.....	0	0	0	0.0	0.0	0.0
Hypocotyls.....	278	0	0	2.5	0.0	0.0
First internodes.....	0	0	0	0.0	0.0	0.0
Primary leaves.....	992	1162	2110	2.9	3.5	7.2
Tops.....	1992	1238	.....	2.9	4.3	.....
Entire plant.....	3262	2400	2110	2.5	2.7	3.1
STARCH, DEXTRIN, AND SUGAR						
Roots.....	215	139	112	1.9	1.3	1.3
Hypocotyls.....	877	233	198	7.9	2.4	2.2
First internodes.....	196	130	442	3.5	2.5	2.3
Primary leaves.....	2292	2324	3546	6.7	7.0	12.1
Tops.....	4465	2016	25	6.5	7.0	2.5
Entire plant.....	8045	4842	4323	6.1	5.5	6.4

plants treated with the acetamide contained a lower percentage of these forms of carbohydrates than did comparable parts of controls. As compared with controls, the plants treated with the naphthalene compounds contained a limited supply of carbohydrate in the form of starch, dextrin, and sugar in tissues adjacent to and below the treated region of the stem.

On the other hand, that portion of the plants above the treatment, including primary and expanded trifoliate leaves of plants treated with the acetamide and primary leaves of those treated with the acid, contained a greater percentage of carbohydrate, mainly in reserve forms (starch and dextrin), than did comparable parts of controls. These results indicate that the expanded leaves of the treated plants were photosynthetically active but any appreciable amount of the synthesize was not transported readily to other parts of the plants, where a relatively low concentration of sugars prevailed. The presence of a relatively low percentage of carbohydrate (starch, dextrin, and sugar) in the roots, hypocotyls, and first internodes of plants treated with the naphthalene compounds could in part be the result of three responses: (1) inhibited leaf expansion; (2) inhibited transport of carbohydrates from the leaves; and (3) increased growth (meristematic activity and secondary thickening in the hypocotyls and first internodes) which would tend to deplete the plant of these forms of carbohydrate.

Nitrogen was present in relatively large amounts in plants treated with naphthalene acetamide and also in those treated with naphthalene acetic acid, as approximately 5 per cent of the solid matter of plants given these respective treatments was nitrogen, while the solid matter in control plants contained only 2.2 per cent nitrogen (tables 4, 5). Distribution of nitrogen throughout plants treated with the naphthalene compounds was unlike that of controls and apparently associated with specific growth responses. That portion of stems near the region to which naphthalene acetic acid was applied developed tumors which were made up largely of meristematic cells (fig. 1), and this portion of the plant contained nearly one-half of the water-soluble nitrogenous compounds, and more than one-third of the total nitrogen in the entire plant. Comparable portions

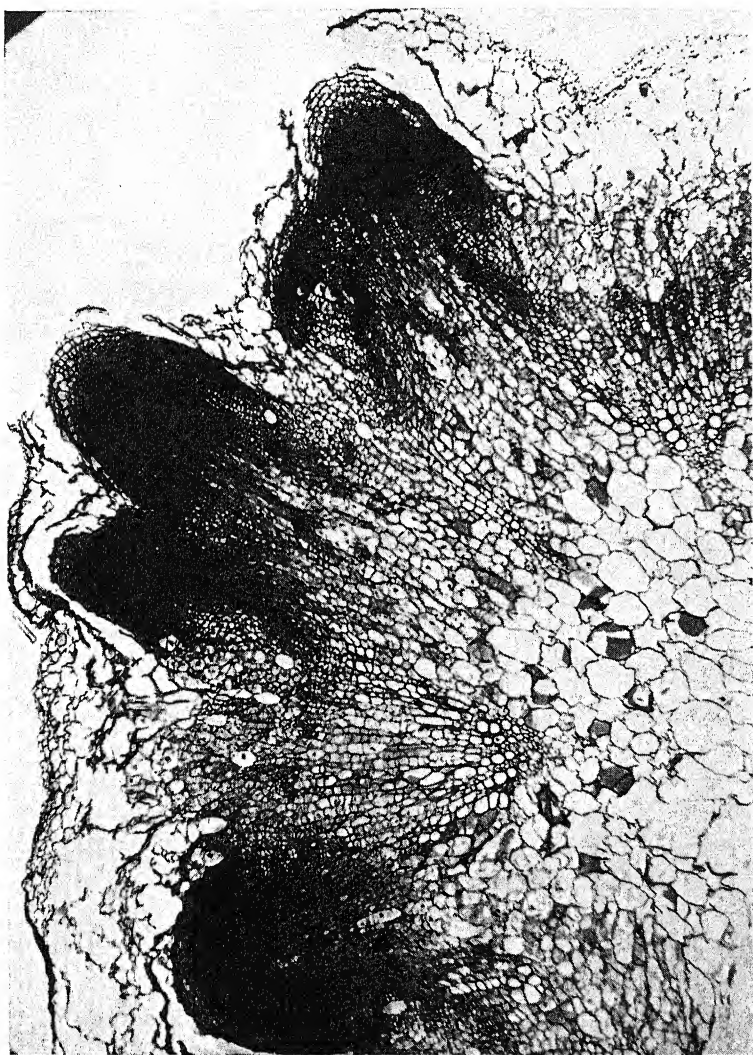


Fig. 1.—Transverse median section of tumor resulting from application (narrow ring) of 2 per cent naphthalene acetic acid-lanolin mixture 96 hours after treatment, showing numerous meristematic cells of the tumor and newly developed roots.

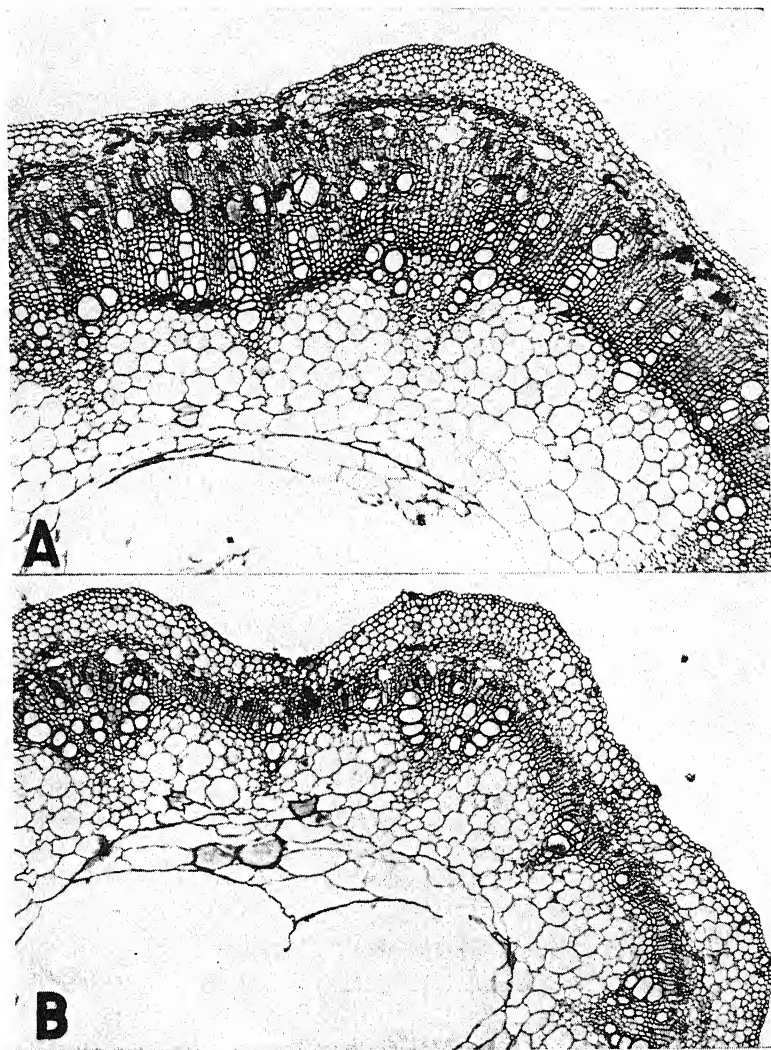


FIG. 2.—*A*, transverse median section through region over which narrow ring of 2 per cent naphthalene acetamide-lanolin mixture had been placed. Section made 216 hours after application. *B*, similar section made from stem treated with lanolin after same period of time. Obvious differences are increased thickening of walls of pericyclic fibers and greater amount of secondary xylem, indicating greater cambial activity in *A*. Cf. fig. 1, same magnification.

of the stems of controls contained less than one-fiftieth of the total nitrogen in the plants.

Tumors did not develop in that portion of stems to which naphthalene acetamide was applied, but instead cambial activity increased and a relatively large number of cells were derived which were characterized by thick walls (fig. 2). A relatively small amount of nitrogen was mobilized in connection with this growth response, as this portion of the stems of plants treated with the acetamide

TABLE 4

AMOUNT OF TOTAL NITROGEN IN PLANTS 12 DAYS AFTER TREATMENT WITH 2 PER CENT MIXTURES OF LANOLIN-NAPHTHALENE ACETAMIDE AND LANOLIN-NAPHTHALENE ACETIC ACID, AS COMPARED WITH OTHERS TREATED WITH LANOLIN

PLANT PARTS	BASIS OF CALCULATIONS								
	PER 100 PARTS (MG.)			PERCENTAGE OF SOLID MATTER			PERCENTAGE AMOUNT IN ENTIRE PLANT		
	LANO-LIN	NAPH-THA-LENE ACET-AMIDE	NAPH-THA-LENE ACETIC ACID	LANO-LIN	NAPH-THA-LENE ACET-AMIDE	NAPH-THA-LENE ACETIC ACID	LANO-LIN	NAPH-THA-LENE ACET-AMIDE	NAPH-THA-LENE ACETIC ACID
Roots and hypo-cotyls.....	457.0	567.1	582.6	2.0	2.8	3.3	7.8	13.1	17.3
First internodes...	108.6	128.4	1223.0	1.9	2.5	6.4	1.9	3.0	36.2
Leaves.....	5309.6	3627.0	1572.6	5.2	5.9	5.2	90.4	83.9	46.6
Entire plant..	5875.2	4322.5	3378.2	2.2	4.9	5.0	.....	.....	.....

contained no more of the water-soluble nitrogenous compounds and only slightly more total nitrogen than did comparable parts of the stems of control plants.

The hypocotyls and roots of plants treated with the acetamide, and also the acid, contained more nitrogen, particularly in the form of water-soluble nitrogenous compounds, than did comparable portions of controls. The presence of a relatively large amount of nitrogen in the hypocotyls of plants treated with the naphthalene compounds was apparently associated with initiation of numerous roots

within the hypocotyls of those treated with the acid, and likewise with initiation of a small number of roots within the hypocotyls of those treated with the acetamide.

TABLE 5

AMOUNT OF WATER-SOLUBLE NITROGENOUS COMPOUNDS AND NITRATE IN PLANTS 12 DAYS FOLLOWING TREATMENT WITH 2 PER CENT MIXTURES OF LANOLIN-NAPHTHALENE ACETAMIDE AND LANOLIN-NAPHTHALENE ACETIC ACID, AS COMPARED WITH OTHERS TREATED WITH LANOLIN

PLANT PART	BASIS OF CALCULATIONS								
	PER 100 PARTS (MG.)			PERCENTAGE TOTAL NITROGEN			PERCENTAGE AMOUNT IN ENTIRE PLANT		
	LANO-LIN	NAPH-THA-LENE ACET-AMIDE	NAPH-THA-LENE ACETIC ACID	LANO-LIN	NAPH-THA-LENE ACET-AMIDE	NAPH-THA-LENE ACETIC ACID	LANO-LIN	NAPH-THA-LENE ACET-AMIDE	NAPH-THA-LENE ACETIC ACID
WATER-SOLUBLE NITROGEN									
Roots and hypo-cotyls.....	219.5	320.3	345.0	48.0	56.5	59.2	12.8	20.5	22.8
First internodes...	56.6	49.9	603.1	52.1	38.9	56.7	3.3	3.2	45.8
Tops*.....	1440.6	1190.4	475.7	27.1	32.8	30.2	83.9	76.3	31.4
Entire plant..	1716.7	1560.6	1513.8	29.2	36.1	44.8	.....	.....	.....
NITRATE NITROGEN									
Roots and hypo-cotyls.....	87.4	144.8	139.0	19.1	25.5	23.9	17.9	30.2	41.1
First internodes...	20.7	12.0	84.5	19.1	9.3	6.9	4.2	2.5	25.0
Tops.....	380.7	322.4	115.1	7.2	8.9	7.3	77.9	67.3	34.0
Entire plant..	488.8	479.2	338.6	8.3	11.1	10.0	.....	.....	.....

\* All portions above first internode.

Application of naphthalene acetic acid to the stems inhibited to a marked degree the extension of internodes and expansion of leaves in the terminal buds during the 12 days following treatment. Comparable applications of the acetamide resulted in similar but less

pronounced effects. The inhibition of growth in the terminal buds of plants treated with the naphthalene compounds was not associated with a limited supply of nitrogen as such in any of the portions of the plants analyzed.

### Summary

1. Application of alpha naphthalene acetamide and alpha naphthalene acetic acid to the stems of bean seedlings affected the total amount and distribution of carbohydrate and nitrogenous compounds in various portions of the plants, and also in the plant as a whole. Some of the chemical responses were specifically associated with the application of the acetamide while another type of chemical response resulted when the plants were treated with the acid. In general the chemical responses were closely associated with growth responses, growth by expansion of leaves and extension of internodes being inhibited in that portion of the plants above the treated section of the stem, while meristematic or cambial activity was stimulated in the treated section and hypocotyls.

2. Tumors, made up largely of meristematic cells, developed in that portion of stems to which naphthalene acetic acid was applied. Analysis of this portion of the stems showed them to contain a relatively high percentage of nitrogen, which was present in the form of compounds that were water-soluble. Increased cambial activity and a relatively large amount of secondary thickening resulted in the treated region of stems following the application of naphthalene acetamide. This portion of the stems contained only slightly more nitrogen than comparable sections of stems of control plants, and a relatively small percentage of the nitrogen present was in the form of water-soluble compounds.

3. Plants treated with naphthalene acetic acid, and also those treated with naphthalene acetamide, contained a lower percentage of starch, dextrin, and sugar in the roots, hypocotyls, and first internodes than did controls. The presence of a limited amount of readily available carbohydrate in these parts of treated plants was associated with the development of a relatively small amount of leaf surface. There was also inhibited transport of carbohydrate from the leaves. Growth responses in the stems were characterized by intensive cambial and meristematic activity, and the initiation of roots

within the hypocotyls. Carbohydrate metabolism and transport was so affected as finally to result in a deficiency in the amount of carbohydrate available in those portions of the plants where growth was stimulated for a period following treatment.

4. There was no evidence of nitrogen deficiency, as the percentage of nitrogen present in all portions of plants treated with the acid—and also those treated with the acetamide—was equal to or greater than that in control plants.

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# INTERRELATIONS IN THE EFFECTS OF BORON AND INDOLEACETIC ACID ON PLANT GROWTH

FRANK M. EATON

(WITH ONE FIGURE)

## Introduction

Some of the symptoms of plants deficient in boron are sufficiently similar to those expected in plants deficient in auxin as to suggest that the role of boron in plant nutrition is closely associated with the formation of auxin and possibly of other plant hormones. The experiments here reported show that indoleacetic acid added to nutrient solutions will partially replace boron.

Plants grown to maturity in sand cultures outdoors have yielded puzzling results in that, with boron concentrations averaging about 0.05 ppm in the nutrient solution, the growth of some fifteen species has been markedly at variance in succeeding summers. In some seasons growth was greatly depressed and there were pronounced deficiency symptoms, whereas in other seasons the same species were normal or nearly so. Analyses of the plant material showed similar concentrations of boron in the normal and deficient plants. Spectroscopic examinations of the plant material for other elements that might have been introduced as impurities replacing boron were negative, as were many culture experiments with elements such as aluminum, gallium, scandium, germanium, and indium, in addition to some of the elements previously tested by BRENCHLEY and WARINGTON (1).

Elongation of the stems, petioles, and roots of plants in minus-boron nutrient solutions is slow or may cease soon after the cotyledons have developed. The rate at which new nodes are formed is affected later. With the tendency for the terminal bud to abort or take on a fasciated appearance, similarly abnormal branches appear in the axils of the leaves. The leaves of cotton and other plants deficient in boron are small, become deeply cupped, and have a peculiarly patched sort of chlorotic mottling. Buckling of the meso-

phyll of boron-deficient leaves indicates a greater depression in the growth of vein structures than of mesophyll. The downward cupping of leaves, as suggested by Dr. H. E. HAYWARD, may reflect also a greater retardation of the growth of the phloem than the xylem elements. Growth of the marginal mesophyll is checked in advance of that adjacent to the main veins, and the formation of lobes of cotton leaves may be partially or wholly repressed. Boron-deficient leaves are usually thickened. Splitting open and corking of veins of boron-deficient plants is sometimes observed. The roots of such plants, as shown in the instance of the pea plant by SOMMER and SOROKIN (2), are short and stubby, and the secondary roots make little growth.

The effects of auxin deficiency on plant growth cannot be described very accurately, since conclusions in many instances must be drawn indirectly or by inference. WENT (4) states that while there is practically no evidence linking leaf-blade growth with auxin, there is considerable circumstantial evidence pointing to the conclusion that auxin specifically conditions petiole and vein growth. Auxin tends to inhibit the development of lateral branches. Root formation is induced by auxin but other hormones have been found essential to root growth.

### Experimentation

In each of the experiments here discussed, Acala cotton plants were grown in Hoagland's solution (5, 5, 2, and 1 millimole per liter respectively of  $\text{Ca}(\text{NO}_3)_2$ ,  $\text{KNO}_3$ ,  $\text{MgSO}_4$ , and  $\text{KH}_2\text{PO}_4$ ) in un-aerated quart mason jars. To this solution, which contained less than 0.01 ppm of boron derived as an impurity in the chemicals, was added 0.1 ppm each of zinc and manganese, and sufficient iron citrate. Indoleacetic acid, when used, was added daily and cumulatively.

The first experiment was conducted in the spring, during a period when most of the days were overcast. There were two storms, each of several days' duration. In this experiment, as shown by table 1 and figure 1, the daily addition of 0.01 ppm of indoleacetic acid to minus-boron solutions resulted in greatly increased leaf development. At the time the measurements were made (the 28th day after transferring the seedlings to the culture solutions), 1 ppm of boron had been present in culture A for only 16 days, whereas indoleacetic

acid had been added daily to cultures C, D, and E for 24 days. In other words, boron was added to a parallel untreated culture only after the first response in root growth from indoleacetic acid had been observed. The leaves on plants in culture E were nearly

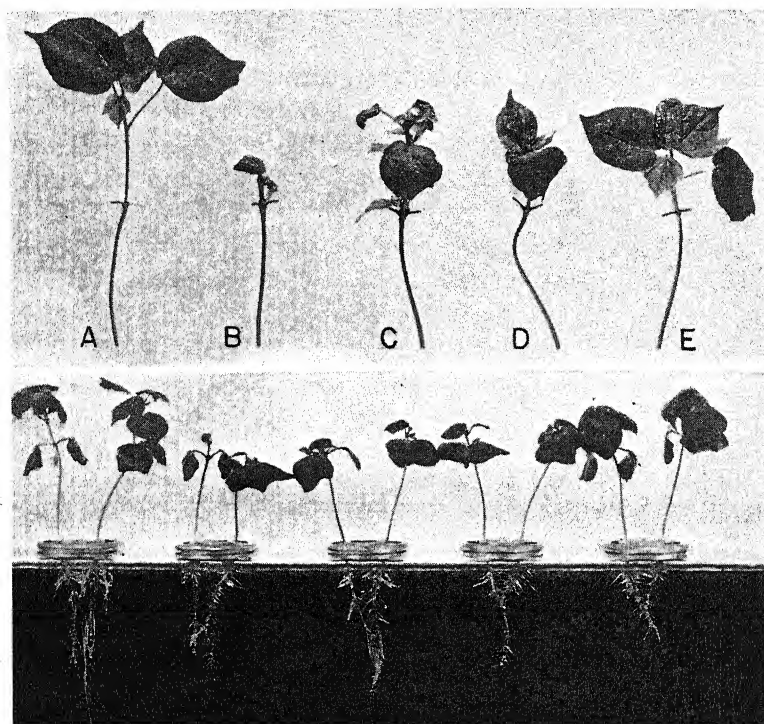


FIG. 1.—Acala cotton plants grown in nutrient solutions. Above, single plant from each culture with cotyledons removed; below, the two remaining plants.

	A	B	C	D	E
Boron added 16 days before photographing, ppm.....	1	0	0	0	0
Indoleacetic acid added daily for 24 days, ppm.....	0	0	0.0001	0.001	0.01

normal in appearance, but it is to be noted that they were not supported in the same outward position from the petioles as those of culture A, which received boron. Internode and petiole elongation was promoted by indoleacetic acid, but in the highest concentration the total length of the internodes was only about half as great as in the plus-boron culture. Indoleacetic acid in a concentration of

0.0001 ppm added daily (culture C) induced root development which for a time appeared normal. Later, however, possibly because of accumulation of an unfavorable concentration of indoleacetic acid and because of retarded development of the leaves, the roots developing near the surface of the solution were shorter than in the plus-boron solution. In the higher indoleacetic acid concentrations (cultures D and E) the roots were short.

In a second experiment that included tomatoes and sunflowers as well as cotton, little or no response to indoleacetic acid resulted.

TABLE 1  
LEAF DEVELOPMENT OF COTTON PLANTS IN SOLUTIONS WITH  
BORON AND WITH INDOLEACETIC ACID

	CULTURE				
	A*	B	C	D	E
Boron, ppm . . . . .	1	0	0	0	0
Indoleacetic acid added daily, ppm. . . . .	0	0	0.0001	0.001	0.01
Leaf areas (exclusive of cotyledons) sq. cm.:					
Plant 1 . . . . .	82	4	9	14	82
Plant 2 . . . . .	78	4	10	11	74

\*In comparing leaf areas, account must be taken of the fact that boron was added to culture A 16 days before the measurements, whereas cultures C, D, and E had been receiving indoleacetic acid for 24 days.

This test, conducted during a period of bright warm days, was discontinued after about the tenth day.

A third experiment was then set up with cotton plants, using four cultures for each treatment. Half of the cultures were placed in a brightly illuminated greenhouse and half in a muslin-covered lath house. The noonday light intensities in the lath house ranged from 500 to 1000 foot candles and in the greenhouse from 4000 to 7000 foot candles.

The plants in the lath house responded to indoleacetic acid in much the same way as they did in the first experiment. The internode elongation in the higher indoleacetic acid concentrations was somewhat better than in the first test, but it was not so good as in the cultures supplied with boron. Any advantages of indoleacetic acid for root development were uncertain or transitory in this ex-

periment. The plants in the brightly illuminated greenhouse showed little response during the early period of the experiment. All the leaves were small and cupped, and no stimulation of root growth was observed. The greenhouse plants were maintained for a month longer than those in the lath house. At the end of this period each successively higher concentration of indoleacetic acid, including a pair of cultures receiving 0.1 ppm, had produced more and larger leaves than the next lower concentration. The total leaf area of the plants receiving 0.1 ppm indoleacetic acid was possibly eight or ten times as great as the plants in "o-boron o-indoleacetic acid" solutions, but none of the leaves were normal. The plants receiving boron were in all respects superior to any of the indoleacetic acid plants and had much greater leaf areas.

Because of the polar movement of indoleacetic acid, concentrations in the foregoing experiments that were sufficiently high to induce favorable responses in the growth of leaves and stems were injurious to the roots. Following a suggestion by Dr. E. J. KRAUS, a subsequent test was conducted in which indoleacetic acid was applied to the leaves and stems of cotton plants in solution with 1 per cent each of lanolin and sodium oleate as a spray three times per week. The concentrations used in this test were probably too low, since a substantial increase in the growth of stems and leaves resulted only from the highest concentration, which was 10 ppm.

Vitamin B<sub>1</sub> and yeast extracts were added, in other experiments, to minus-boron solutions alone and in conjunction with indoleacetic acid. These substances did not improve growth nor lessen the severity of boron deficiency symptoms.

### Discussion

These experiments provide evidence that boron as an element essential to the growth of plants can in some measure be replaced by indoleacetic acid. In no test, however, were the results obtained with indoleacetic acid equal to the responses that followed additions of boron to nutrient solutions. The findings point to the conclusion that at least one of the functions of boron in plant nutrition is intimately related to the formation of plant hormones. Any interpretation of the results must take into account the experimental

difficulties associated with the movement of indoleacetic acid into the plant and to the active tissues in concentrations neither too high nor too low. Furthermore, possible distinctions must be recognized between the effects on plant growth of indoleacetic acid and auxin.

VAN OVERBEEK (3), by *Avena* coleoptile studies, has found that to some extent indoleacetic acid is inactivated by light, but to a lesser extent than is the case with auxin *a*. This fact may serve to account for some of the differences in responses of cotton plants under high and low illumination.

The inquiries on the comparative reactions of cotton plants to indoleacetic acid and boron had the work mentioned in the introduction as its background. Inasmuch as the evidence indicates some replaceability of boron by acetic acid and the importance of light and possibly of temperature in the reactions, a possible advance has been made in an understanding of the role of boron. A clue is thus provided as to why like amounts of available boron, both in culture solutions and it seems also in the field, may be effective to different degrees in different seasons with varied climatic conditions.

### Summary

Experiments with young cotton plants show that indoleacetic acid will to some extent replace boron as an element essential to the growth of root, stem, leaf vein, and other leaf blade tissues. The results suggest that boron is essential to the formation of auxin in plants.

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## LIGHT STABILITY OF AUXIN IN AVENA COLEOPTILES

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### Introduction

Auxins *a* and *b* and their lactones, when extracted from plants, are not inactivated by light of wave lengths longer than 4000 Å (6, 16). If a decreased growth of plants in light as compared with those in darkness is considered to result from inactivation of auxin (2, 7, 10, 11, 12), then wave lengths longer than 4000 Å do inactivate auxin when it is inside the plant. VAN OVERBEEK (9) assumed that this growth decrease was due to decreased sensitivity of the growing cells to auxin, since in transport experiments destruction of auxin was not found. WENT and THIMANN (17) have suggested that light may inactivate only the bound and not the transportable auxin.

If this latter suggestion were true it would give a valuable argument in support of the hypothesis that auxin exists in different states inside the plant, namely, as a free-moving or transportable auxin, and as bound auxin (17). Free-moving auxin can be obtained from plants by placing the parts to be investigated with their basal cut surfaces on agar, when such free-moving auxin will diffuse into the agar and can then be quantitatively determined by the *Avena* test. The bound auxin comes out of the cells after killing them. It is obtainable by extracting them with organic solvents, for example, ether. This extraction presumably removes both free and bound auxin. To test this suggestion by WENT and THIMANN of differential inactivation by light of free and bound auxin, bound auxin was extracted from plant material grown in complete darkness and from comparable material after exposure to light. With the same kind of material, "transport" experiments with free-moving auxin were likewise carried out in light and in darkness. Precautions were taken to exclude all light, even the weakest red light, from the plants grown

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in darkness. This precaution was necessary as experiments (13) have shown that the orange darkroom light is sufficient to decrease auxin production 33 per cent in *Avena* coleoptile tips.

### Material

It was first thought that *Raphanus* would be a good experimental plant to investigate the destruction of auxin inside the plant by light, but a growth inhibitor completely masked the auxin present (8), so that another experimental plant had to be chosen. Seedlings of Victory oats from Svalöf, Sweden, were therefore grown in the standard manner (17), with the exception that immediately after planting they were placed in complete darkness. Later, extracts with ether according to VAN OVERBEEK (14) were made. The test plants for the auxin determinations were grown with occasional orange light, as is usual for the *Avena* test.

### Results

#### EFFECT OF LIGHT ON AUXIN DURING ETHER EXTRACTION

Results of preliminary experiments showed that during the course of an ether extraction the extracted auxin was not inactivated by light. In these experiments *Avena* plants were grown for four days in total darkness, at 85 per cent relative humidity and 25° C., in a mixture half sand and half peat moss. The mesocotyl, coleoptiles, and primary leaves were then extracted as four separate lots with peroxide-free ether while still in total darkness. Twenty-four hours later the ether from two of the lots was evaporated off in darkness while with the other two it was evaporated off in daylight. The auxin was taken up from the ether extract by evaporating the last 1-2 cc. of ether to dryness on hot 1.5 per cent agar. The agar was then tested for auxin by the *Avena* test.

To facilitate the ether extraction in the dark, a few modifications of the method of VAN OVERBEEK (14) were made. A well was made in the center of the bottom of a 125-cc. Erlenmeyer flask by blowing an opening in the bottom and fusing a shell vial (1.2×3 cm.) over it on the outside. When used in extractions, the desired volume of agar was placed in the well and the ether to be evaporated added. As the ether evaporated the extracted auxin was taken up

by the agar. It was found essential to wash down the walls of the flask several times with purified ether to prevent loss of auxin on the walls. After evaporation, the agar was removed from the well, solidified, and cut into small blocks for application to the *Avena* test plants. With these modifications it was necessary only to use momentarily a small point of dim red light when separating the ether from the water layer of the extracted material. The data from four experiments are given in table 1. No difference in the amount of

TABLE 1

EFFECT OF LIGHT DURING ETHER EXTRACTION OF AUXIN FROM AVENA COLEOPTILES; EXTRACT TAKEN UP IN 0.283 CC. 1.5 PER CENT AGAR  
IN ALL INSTANCES

EXPERIMENT NO.	No. TEST PLANTS PER DETER- MINA- TION	AUXIN EXTRACTED						AUXIN IN EX- POSED PLANTS AS PER- CENTAGE IN UN- EXPOSED
		UNEXPOSED			EXPOSED			
		ACTUAL NEGA- TIVE CURVA- TURE (DE- GREES)	AMOUNT EX- TRACTED (GM.)	CURVA- TURE PER GM. (DE- GREES)	ACTUAL NEGA- TIVE CURVA- TURE (DE- GREES)	AMOUNT EX- TRACTED (GM.)	CURVA- TURE PER GM. (DE- GREES)	
161.....	12	18.3	1.355	13.6	13.0	0.833	15.6	115
162a.....	12	16.0	0.843	18.9	18.7	1.054	17.7	93
162b.....	12	16.0	0.978	16.4	17.2	1.077	16.0	97
164.....	18	15.3	0.909	16.9	14.0	0.819	17.1	101
Average.....				16.5			16.6	101

extracted auxin was found whether the ether evaporation was carried out in darkness (except for momentary use of the red light) or in diffuse white light. It was assumed therefore that there was no destruction of auxin when the ether extract containing auxin was handled in a laboratory receiving only indirect daylight.

#### EFFECT OF LIGHT ON ETHER-EXTRACTABLE AUXIN IN PLANT

The procedure of the experiments on the effect of light on extractable auxin was to make ether extractions of auxin from *Avena* coleoptiles exposed to light and from unexposed coleoptiles (con-

trols). The auxin thus extracted, containing both free and bound auxin, was measured by the standard *Avena* test.

In some experiments only the coleoptile was extracted while in others the coleoptile and the primary leaf were used. In some in-

TABLE 2  
EFFECT OF LIGHT ON ETHER-EXTRACTABLE AUXIN

EXPERIMENT NO.	NO. TEST PLANTS PER DETERMINATION	EXPOSURE TO LIGHT (SECONDS)	VOLUME AGAR FOR EACH EXTRACT (CC.)	AUXIN EXTRACTED				AUXIN IN EXPOSED PLANTS AS PERCENTAGE IN UNEXPOSED	NOTES
				UNEXPOSED		EXPOSED			
				CURVATURE (DEGREES)	WEIGHT (GM.)	CURVATURE (DEGREES)	WEIGHT (GM.)		
132...	12	1	0.3	5.9	1.089	5.2	1.170	82.2	Tip 1.5 cm. of coleoptile extracted; exposed to sunlight after removal from plant
19....	24	1	0.5	10.8	1.202	12.7	1.715	81.0	Same as 132
25....	24	5	0.4	13.0	1.183	14.3	1.450	89.2	Same as 132
8....	12	5	0.3	6.5	0.782	4.9	0.841	69.8	Coleoptile and leaf extracted; tip cm. of plant extracted; exposed while on plant
14....	36	10	0.4	9.5	1.211	9.1	1.705	67.7	Same as 132
45....	12	10	0.3	12.3	1.047	13.9	1.309	89.6	Same as 132
36....	20	10	0.5	5.5	1.400	11.1	2.727	109.0	Same as 132
167....	20	30	0.57	11.0	2.654	8.1	2.632	83.0	Same as 132
8....	12	30	0.3	6.5	0.782	5.1	0.865	70.8	Exposed while on plant
6....	24	60	0.4	11.7	0.960	13.2	1.380	78.8	Tip 2 mm. of coleoptile removed; only coleoptile extracted; exposed while on plant
5....	12	60	0.3	22.1	1.002	20.8	1.320	72.6	Tip cm. of coleoptile extracted; exposed while on plant
47....	24	100	0.4	14.0	1.623	17.1	1.648	120.0	Same as 132
8....	12	600	0.3	6.5	0.782	5.3	0.839	75.1	Exposed while on plant
1....	12	45 (min.)	0.4	8.3	0.720	8.8	1.430	53.0	Only coleoptile exposed to sunlight while on plant
15....	24	10	0.5	10.5	2.348	9.7	3.300	64.5	Same as 132, but exposed at 6000 ft. elevation
159....	12	60	0.28	12.3	1.447	8.4	1.045	94.1	Mesocotyl and coleoptile extracted; exposed 1½ ft. from 60 watt Mazda bulb on 110 v. AC
169....	22	60	0.57	10.9	4.216	7.9	4.100	76.0	Same as 159
Average								81.0 ± 3.3	

stances the tip millimeter of the coleoptile was removed. Most of the plants extracted were grown as for the regular *Avena* test, illuminated during the first 36 hours of germination by a red light which inhibits growth of the mesocotyl. In experiments 159, 161, and 169 the plants were grown in complete darkness. In the first

experiments the coleoptiles were exposed to direct sunlight while they were still attached to the plant; in later experiments the coleoptiles were removed, placed on moist filter paper in a shallow glass dish, and then exposed to light. The material was weighed and immediately extracted. The controls in the dark in all experiments were always kept in a light-tight box directly beneath the plants being exposed to light, so that temperature conditions were nearly the same for both sets of plants. Data of fifteen experiments, summarized in table 2, show that light exposures as short as one second decreased the amount of ether-extractable auxin. The data are too variable to draw any further conclusions regarding the effects of various types of illumination (sunlight, electric incandescent light, etc.), but the fact that less auxin can be extracted from plants which have been illuminated with white light cannot be doubted, even though in two out of seventeen cases an increase was obtained. Statistically the decrease of extractable auxin is significant.

#### EFFECT OF LIGHT ON FREE-MOVING AUXIN

The auxin which diffuses from living plant tissues is presumably being transported through the plant and is spoken of as free-moving auxin. Light apparently does not decrease the amount of free-moving auxin in *Avena* coleoptiles, in contrast to its effect on bound auxin. Free-moving auxin is transported through *Avena* coleoptiles at a rate of 10-12 mm. per hour. Accordingly, with sections of *Avena* coleoptiles 5.25 mm. long, auxin applied at the top should move through them in approximately 30 minutes. Sections this length were used in the following experiments. These sections were allowed to stand with their basal ends on wet filter paper for at least 30 minutes and then were placed upright on 1.5 per cent agar blocks. On the tops of the sections agar blocks were placed. All the top agar blocks contained the same concentration of auxin *a* or *b*, which had been extracted from corn meal (12). (Auxin *a* or *b* naturally occurs (4) in *Avena* coleoptile tips.) The sections were kept in the dark, in petri dishes lined with moist filter paper. After 30 minutes they were exposed to sunlight for one minute and then allowed to remain in the dark for an additional 29 minutes. At the end of this time the amount of auxin in the lower agar block was determined in both the exposed plants and the non-exposed controls. Using this technique,

it was presumed that the auxin had just begun to diffuse out of the lower end of the section and was present throughout its entire length at the time of its exposure to light. Twenty-nine minutes later the auxin that was at the top of the section of the coleoptile at the time of its exposure to light would be moving out of the section into the lower agar block. Thus if there had been any decrease in the amount of the transported auxin, a comparison of this experiment with an unexposed, control experiment would be expected to

TABLE 3  
EFFECT OF LIGHT ON FREE-MOVING AUXIN GIVEN  
AS DEGREES NEGATIVE AVENA  
COLEOPTILE CURVATURE

EXPERIMENT NO.	NO. TEST PLANTS PER DETERMINATION	CURVATURE (DEGREES)	
		UNEXPOSED	EXPOSED
171.....	{ 11	4.5	5.2
	{ 9	4.5	4.2
	{ 10	4.8	4.3
	{ 12	5.5	4.3
172.....	{ 9	6.4	4.9
	{ 9	3.6	4.0
	{ 7	3.4	4.2
173.....	{ 12	5.0	6.1
	{ 12	6.8	6.9
	{ 12	5.3	6.2
Average.....	.....	5.0	5.0

show less auxin passing through the section. This was not found to be the case (table 3). The same amount of auxin was present in the lower agar block in both the exposed and the unexposed sections. One experiment of this type was continued over 2 hours by exposing the same sections to light for one minute after  $\frac{1}{2}$ , 1, and  $1\frac{1}{2}$  hours in the dark (experiment 173; table 3).

### Discussion

These experiments show that in the *Avena* coleoptile the distinction between free-moving or diffusible auxin and the auxin inside cells, or bound auxin (17), is based on an actual difference in the condition of the auxin. The amount of auxin moving through an

*Avena* coleoptile section is not affected by light, whereas the ether-extractable auxin is partially inactivated even by short exposure to white light.

Since the ether-extracted auxin is probably a combination of free-moving and bound auxin, and free-moving auxin is light-stable, it follows that it is the bound auxin that is inactivated by light. This conclusion had previously been drawn from indirect experimental evidence. The experiments reported here give direct evidence, however, and as the determinations were all made on the same material, *Avena* coleoptiles, the reaction of the bound and free-moving auxin to white light could be compared. Such had not previously been the case.

KÖGL, KONINGSBERGER, and ERXLEBEN (5) and C. KONINGSBERGER (6) have shown that radiation below 4000 Å wave length inactivates only the lactone form of the crystalline auxin *a* or *b*. The free acid and the lactone form of auxin *a* or *b* are stable in visible light. This lactone in concentrated alcoholic solution is in equilibrium with the free acid form of auxin *a* at a ratio of about 50 per cent lactone and 50 per cent free acid (6). In the plant, however, visible radiation, which failed to affect crystalline auxin or its lactone, causes an inactivation of bound auxin. This may indicate that in the plant there is another substance present, an activator, which is required before visible radiation can inactivate the auxin. This activator possibly would be a carotenoid (1, 7, 15).

From the foregoing considerations it seems evident that while the auxin is being transported, this activator is unable to function. It also seems evident that inactivation does not occur through the lactone form, as suggested by KÖGL and KONINGSBERGER. In this regard also the mean percentage inactivation which could be expected would be 50 per cent if all the lactone were destroyed; furthermore, since auxin and lactone are in equilibrium in solution, one would expect a rapidly increasing percentage inactivation if the illumination continued for 45 minutes or longer. This was not found (table 2). The experiments do not support the hypothesis that the inactivation of auxin in plants by light is directly by way of lactone; they suggest rather that auxin is inactivated by light when it is in combination with some cell constituent.

While these experiments show possibly for the first time by direct determination that the bound auxin inside the plant is inactivated by light, others had already found that light does not decrease the amount of transported auxin. VAN OVERBEEK (9) has shown that in light 106.5 per cent auxin is transported through sections of *Raphanus* hypocotyl sections, if transport in the dark is considered as 100 per cent. The data from tables 9 and 10 of DU BUY (3) give 95.5 and 97 per cent transport of auxin in the illuminated sections when compared with the dark sections. These figures, as our own, are so close to the values for sections in the dark (100 per cent) that the conclusion seems justifiable that light does not decrease the amount of free-moving auxin.

### Summary

Auxin, once it has been extracted from plant tissues, is stable in light. As shown by ether extractions, 19 per cent of the auxin inside the plant is inactivated by sunlight or incandescent electric light (23.5 per cent inactivated, omitting negative cases). There is no corresponding decrease in the case of free-moving auxin.

This work was done at the California Institute of Technology, Pasadena, California.

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## FLUORESCENT LAMPS AS A SOURCE OF LIGHT FOR GROWING PLANTS

(WITH ONE FIGURE)

A number of sources of artificial light have been employed in growing plants under controlled conditions. For various reasons, most of these have proved unsatisfactory. Carbon arc lamps give light of high intensity and good quality, but are costly to install and maintain. Other sources of light are poorly balanced spectrally for best growth, give off large quantities of heat, and usually necessitate the use of cooling systems when utilized in closed chambers.

We have known for some time that Dr. D. R. HOAGLAND (University of California ) and Dr. F. W. WENT (California Institute of Technology) have been successfully using lamps of the fluorescent type in some of their experiments. Recently we have grown plants using 30-watt fluorescent Mazda lamps of both white and daylight types but of no other colors. These lamps were arranged so as to give an intensity of approximately 600 foot-candles at the leaf surface and were the sole source of light. They were operated 16 hours each day. For purposes of comparison, some plants were grown with ordinary winter daylight and others in winter daylight supplemented (beginning at 4:30 P.M.) by 9½ hours of approximately 60 foot-candles of light from an incandescent filament lamp. Cabbage, cocklebur, corn, kidney bean, Biloxi soybean, tobacco, and tomato were used. Plants under the fluorescent light grew most rapidly, had sturdiest stems, shortest internodes, largest and most numerous leaves of the deepest green color, and greatest fresh and dry weights. In general they appeared more like plants grown in summer sunlight than did those plants grown under either of the other two conditions of illumination (fig. 1; see p. 716).

Since these lamps give off a negligible amount of heat, they can be placed very close to the experimental material. They are much more economical and efficient in operation than incandescent filament lamps, and can be variously arranged in kind, number, and position so as to give different qualities and intensities of light. All points considered, these lamps have been found the best source of artificial light for growth of plants when used as the only source of light or as a supplement in winter in regions where daylight is of inferior quality and low intensity.—AUBREY W. NAYLOR AND GLADYS GERNER, *Department of Botany, University of Chicago.*



FIG. 1.—Response of plants to different types of illumination. *D*: ordinary winter daylight; *DS*: ordinary winter daylight supplemented by  $9\frac{1}{2}$  hours of incandescent filament light of 60 foot-candles intensity; *FL*: fluorescent light of approximately 600 foot-candles intensity for 16 hours per day as sole source of illumination. I, tobacco; II, cocklebur; III, tomato; IV, cabbage; V, kidney bean; VI, corn.

## CURRENT LITERATURE

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*Les Fusarium et cylindrocarpon de l'Indochine.* By FRANCIS BUGNICOURT. Encyclopédie mycologique XI. Paris: Paul Lechevalier, 1939. Pp. 206. Illustrated. 165 fr.

*Fusarium* and its fairly recent segregate *Cylindrocarpon* constitute a group of fungi of great importance, as well as one of great difficulty. The perfect stages of all species, so far as known, are members of the Hypocreales, but even if all were known, it would still be essential to consider the imperfect stages, the aspect under which these forms commonly present themselves in nature and in culture. The present volume gives detailed descriptions, cultural characteristics, and illustrations of some forty species and varieties of the two genera, isolated from various substrata in Indo-China over a period of 8 years, of which two varieties of *Fusarium* and nine species of *Cylindrocarpon* are described as new.

Every species and variety treated is illustrated by outline drawings of micro- and macroconidia and chlamydospores, if present; photomicrographs of most of them are reproduced on the six collotype plates and growth characters used in classification are fully shown on the four colored plates. The classification of *Fusarium* follows the well-known treatment of WOLLENWEBER and REINKING, the species included representing nine of the sixteen sections of those authors. It is unfortunate that the original numbering of the sections is not retained, while the lack of a complete index, including synonyms, is to be regretted. The present work is a useful supplement to the standard monograph mentioned, particularly as extending our knowledge of tropical pathogenic fungi and their host relationships.—G. W. MARTIN.

*Growing Plants in Nutrient Solutions.* By WAYNE I. TURNER and VICTOR M. HENRY. New York: John Wiley & Sons, 1939. Pp. xiv+154. Figs. 29. \$3.00.

The popularity of solution culture gardening among amateurs has led to the publication of many books dealing with this pastime. As a commercial method of plant production, especially for greenhouses, solution culture offers a number of advantages which offset the original expense of installation of the necessary machinery. It seems probable, therefore, that commercial nutrient solution culture will gradually come into its own, while the popular fad is bound to decline.

For those who contemplate the conversion of greenhouses to the nutrient culture technique, this book offers much valuable information. The general

principles are discussed, the commercial advantages, and the construction changes required to convert a greenhouse from soil to the new method. Directions are given also for small-scale experimental units for those who may profit from preliminary investigations of their plant nutrition problems. Information required for the intelligent preparation of nutrient solutions, mathematical computations, sources of needed materials, and accepted formulae are presented simply and clearly. The book discusses the general physiological reasons for controlled culture methods; the problem of the essential elements and their functions in growth; methods of testing soils and foliage for mineral deficiencies; a key useful in diagnosis of deficiency symptoms; and the general cultural conditions to be met in successful commercial application of nutrient culture methods.

This work deserves to be classed among the better books dealing with solution culture methods. It is well written, fairly accurate, and does not misrepresent the subject nor attempt to exaggerate the importance of this new-old technique, which has been used by plant physiologists for nearly three-quarters of a century.—C. A. SHULL.

*Submikroskopische Morphologie des Protoplasmas und seiner Derivate.* By A. FREY-WYSSLING. Protoplasma-Monographien, Band 15. Berlin: Gebrüder Borntraeger, 1938. Pp. x+317.

Applying morphological description to the molecular structure of protoplasm, FREY-WYSSLING has written an interesting and thought-provoking book. The domain of submicroscopic and molecular structure in biology is shown to fit between the cell theory and the atomic theory. It bridges the gap between the descriptive morphology of the cytologist and the atomic and molecular concept of the physicist.

As a foundation for the submicroscopic morphology of the protoplasm, the author presents concise discussions of: the phase theory and its relation to colloidal chemistry; the structural theory of atoms and molecules—their dimensions and spatial orientations as determined by X-ray diffraction measurements and their physical properties resulting from these characteristics; and the micellar theory. In the discussion of the micellar theory, gel structure is discussed at length, particularly as it is revealed by polarized light studies. Applying these theories to protoplasm, a description of the molecular morphology is conceived.

In the discussion of the cytoplasm, a "cohesion point" theory is suggested to build together the protein, lipoid, and phosphatide building units into a structural entity. The nucleus is considered from a similar viewpoint. Chloroplasts and their structural building units are discussed. Double refraction phenomena and microscopic structure are used to develop chloroplast structure from a molecular standpoint. The remainder of the book is concerned with the submicroscopic structure of protoplasmic derivatives. These are classified

as either structural substances (cellulose, chitin, keratin, etc.) or as reserve materials (starch, protein, crystals, etc.).

Throughout, the physiology discussed is mainly that of plants, although there are sections on the molecular structure of muscle during muscle contraction; on silk; on keratin; etc.

This book is essentially an application to protoplasm of the same concepts applied by the author to the cell wall in his previous work, *Die Stoffausscheidung der höheren Pflanzen*. It is well illustrated with diagrams, pictures, and tables. It contains a very complete bibliography of references not commonly readily available to biologists.—W. S. STEWART.

*The Microscope*. By R. M. ALLEN. New York: D. Van Nostrand Co., 1940. Pp. viii+286. Figs. 82, pls. 72. \$3.00.

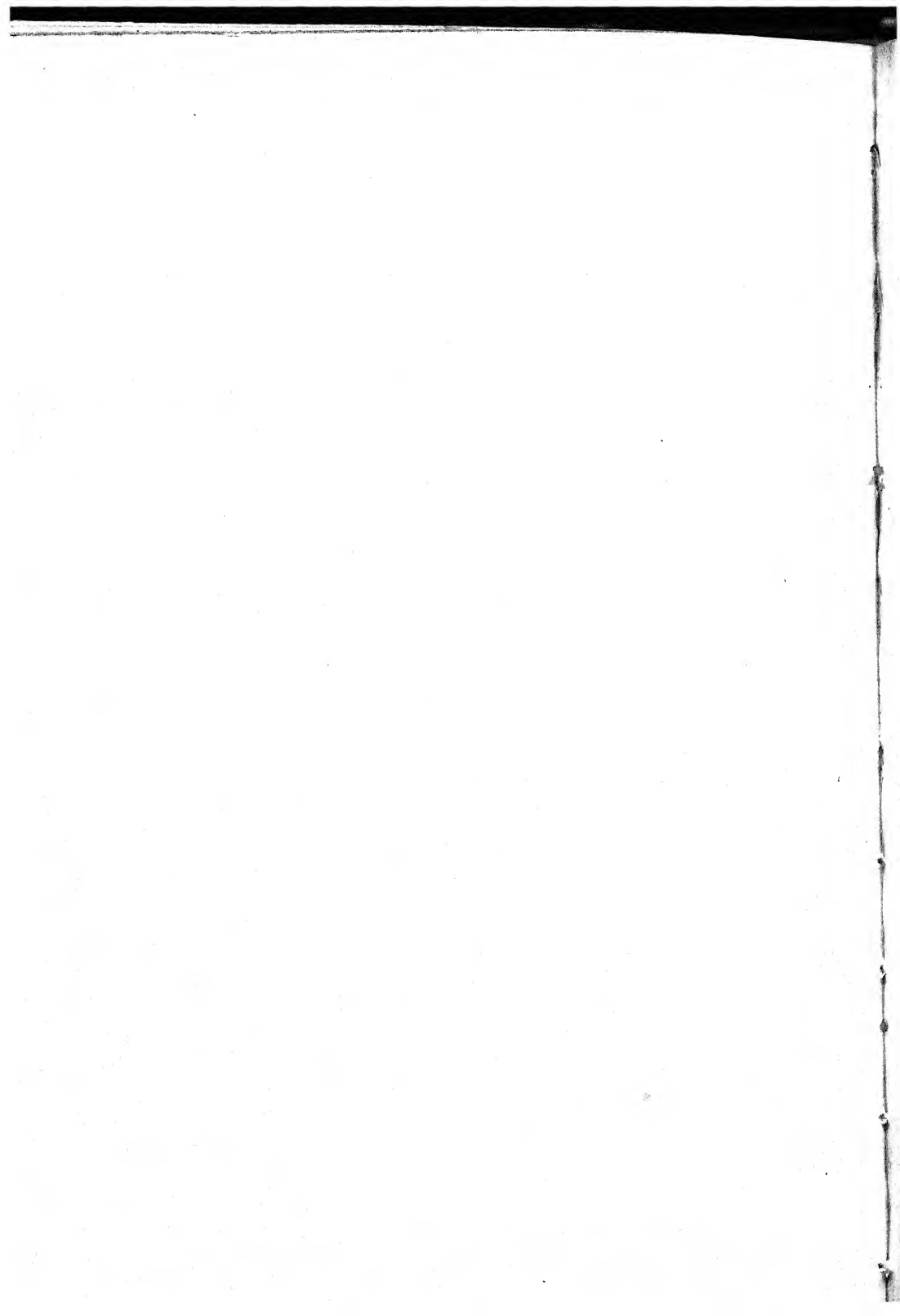
The work is a generalized treatise, which outlines the history of the microscope, its gradual improvement with time, descriptions of modern instruments and how to use them most effectively. A chapter on preparation of material for microscopical examination applies mainly to materials other than plant tissues.—E. J. KRAUS.

*A Manual of the Liverworts of West Virginia*. By NELLE AMMONS. Notre Dame, Ind.: University Press, 1940. Pp. 164. Illustrated. \$1.75. (Amer. Midland Nat. 23: 3-164. 1940.)

Although this manual covers a limited and artificial geographic area, its use in bryological studies will undoubtedly extend to all states surrounding West Virginia, and many others. Keys, partial descriptions, comments, and illustrations for 111 species representing 56 genera constitute most of the book. The characteristics of liverworts and methods for their collection and preservation are presented lucidly.

It is to be regretted that *Plagiochila virginica* Evans is listed as not treated in this manual (Appendix I). The type of this plant, together with the rest of Dr. MILLSPAUGH's collections, is in the Herbarium of Field Museum, Chicago, where perhaps it may have been unavailable for many years. Citation of collectors with dates of at least the rarer species would have enhanced the value of the book.

Since the arrangement of genera is in accordance with the latest interpretations of a phylogenetic sequence, this manual is a very useful compilation of hepatics.—P. D. VOTH.



# THE BOTANICAL GAZETTE

June 1940

## PERMANENT CHANGES OF CHLOROPLASTS INDUCED BY X RAYS IN THE GAMETOPHYTE OF POLYPODIUM AUREUM

LEWIS KNUDSON

(WITH FORTY-SIX FIGURES)

### Introduction

In an investigation on the effects of X rays on growth response and physiological processes in the gametophytic stage of *Polypodium aureum*, certain extraordinary changes were infrequently noted in the chloroplasts of protonema and prothallia derived from spores exposed to relatively high intensities of irradiation. So remarkable were these modifications, and so significant relative to important problems in genetics and physiology, that it appeared desirable to give especial attention to this phase of the problem.

While data on germination and growth were obtained and various extraordinary modifications of protonema and prothallia obtained which will be reported subsequently, the primary problem became a study of these plastids. It was believed that such study could contribute not only to a fuller knowledge of the structure of the plastid, but—if a sufficient number of plants could be obtained—a real contribution could be made to the question of the individuality of the plastid, its continuity from generation to generation, and plastid inheritance. Is the change in the plastid due to genic factors or is it due to cytoplasmic changes and independent of chromosomal

alterations and aberrations? It is recognized, of course, that modification of the plastid may be determined by genic factors, and it has been demonstrated for both animals and plants that the frequency of heritable alterations is markedly increased by X rays and that often associated with these are chromosomal alterations and aberrations (28, 3, 4).

Botanists in general are inclined to the view that plastids arise only from pre-existing plastids, so that there is a continuity from one generation to another. WEIER (30) has recently summarized the literature on this problem, and concludes that the "most careful work indicates a continuity of the chloroplast." SHARP (25), recognizing definite continuity of plastids in certain algae, maintains that the genetic continuity of plastids in general still remains to be proved. WEIER emphasizes the contributions of DE VRIES, RENNER, and others on that type of plastid inheritance which indicates genetic continuity. From the viewpoint of genetic continuity, the work of LANDER (16) on the chloroplasts in *Anthoceros* is significantly favorable. STONE (29) among recent investigators concludes that plastids arise *de novo*.

It is generally recognized that in meristematic cells the plastids exist as minute bodies, to which a variety of names have been given: mitochondria, chondriosomes, plastid primordia, archiplasts, and proplastids. The last term, suggested by RANDOLPH (23), is preferred by BOWEN (2), who is able to differentiate these proplastids from similar appearing bodies. In meristematic cells it is not uncommon to find these proplastids grouped about the nucleus, as was observed by RANDOLPH (23) in corn. The plastids are in the cytoplasm of course. They grow and divide, and their shape and size may, within limits, vary with nutrition of the cell.

That properties of the cytoplasm are modified by X ray treatment has been demonstrated. It is also probable that such modifications may be associated with the increased ionization and molecular excitation that results from X rays (4, 5, 6). With chemical or physical-chemical changes in the cytoplasm itself there might be induced changes in the plastids, or changes in the plastids could occur because of direct action of the X rays. The point to be emphasized is that these changes could occur without involving chromo-

somal behavior. If such changes should persist and if there is continuity of plastids from generation to generation, then mutations in plants could occur without chromosomal changes, especially mutations involving growth and yield.

Entirely aside from the problem of inheritance and plastid individuality, the extraordinary modifications noted in the course of this work seem to offer material of especial value for studies in plastid structure and behavior, and for studies on the relation of size, form, and structure to photosynthetic efficiency. Here in a single species occur distinct types of chloroplasts. The questions arose, will these modifications persist, and can these prothallia be vegetatively increased so that an adequate stock of material will be available for study?

Altogether fifty-one prothallia with marked plastid changes were isolated. Of these only twenty-seven survived, but fortunately these included all the plastid types that had been observed. Fortunately also it proved a relatively simple problem to multiply the prothallia by vegetative reproduction, and at present an abundant stock of material is available. It is the purpose of this first paper to describe the methods employed and to describe the plastid types that have been produced. Subsequent papers will deal with the transmission of these plastids to sporophyte and new gametophyte generations.

#### Material and methods

The fern was selected as the plant material because it produces minute spores which are simple in structure. Any effect induced by X rays would be direct and not secondary, as is possible when seeds are used. Furthermore, the spores could be germinated under pure culture conditions and a large number of plants could be grown in a single tube in the entire absence of insects and microorganisms. Any number of plants could be removed from the tube at will for microscopic examination without damaging the remaining plants of the culture. *Polypodium aureum* (*P. glaucum*) (*Phlebodium aureum*), commonly known as the hare's foot fern, was selected because it produces spores at all seasons of the year and in large quantity. This plant is native to southern Florida and tropical America. While the plant is epiphytic, it grows readily in soil. Both sex organs are

borne on the same prothallium. As is well known, the spore is of the gametophytic generation and has therefore the haploid complement of chromosomes.

**IRRADIATION.**—For irradiation the spores were placed in a small glass container made by cementing a cover glass to a glass cylinder (diameter 1.8 cm.) such as is used in the preparation of a Van Tieghem cell. The bottom of this cell was covered to a depth of about 1 mm. with spores. About ten containers were placed under the X ray tube at a distance of 12.5 cm. from the target. The tube was a Coolidge type 5-30, operated at 78-80 k.v. with a current of 4 milliamperes. Radiation was not filtered. The irradiation was intermittent; that is, the material was irradiated for one minute, with a pause of one minute. Under the conditions described the intensity of the radiation was 1000 r units per minute.

The intensity was measured by means of a Fricke-Glosser dosimeter using chamber no. 178 for unfiltered radiation. The dosimeter had been calibrated at the factory just previous to its use.

**NUTRIENT SOLUTION.**—The nutrient solution was solution B as used in my investigations on the germination of orchid seed (11), except that it was necessary to adjust the solution to a pH 5.7 to 6. This was done by using  $K_2HPO_4$  instead of the monobasic potassium phosphate. No attention was paid to the addition of manganese, boron, copper, or zinc since these were present as impurities in the chemicals used. In certain of the experiments glucose or sucrose was added to the extent of 2 per cent.

This nutrient medium was heated in an autoclave at 10 pounds' pressure until the agar was "dissolved," and then approximately 30 cc. of the culture medium added to large culture tubes ( $21 \times 2.5$  cm.). The tubes were provided with cotton stoppers and autoclaved for 15 minutes at 15 pounds' pressure, after which they were placed in an inclined position to slope the agar medium.

**STERILIZATION OF SPORES.**—Following irradiation, the spores were placed in small test tubes  $10 \times 1$  cm. and the tubes nearly filled with the filtered solution of calcium hypochlorite. The tube was closed by means of the forefinger and shaken until each spore was wetted. A 10-15 minute treatment was sufficient to free the spores of microorganisms. This is essentially the method developed by

WILSON (31), except that only 10 gm. of calcium hypochlorite was used to 125 cc. distilled water.

Previous experience had indicated that fern spores could be treated for considerable time with the calcium hypochlorite without exhibiting evidence of injury. Nevertheless it was thought desirable to determine definitely the effect of the duration of treatment. The usual nutrient solution was used, to which was added 2 per cent glucose to facilitate growth of any microorganism that might be present. All treatments were in triplicate. The exposures to calcium hypochlorite varied from 10 to 50 minutes. The results on germination and growth are shown in table 1.

TABLE 1  
INFLUENCE OF TIME OF TREATMENT WITH HYPOCHLO-  
RITE ON GERMINATION AND GROWTH; DURATION  
OF EXPERIMENT, MARCH 30 TO APRIL 14

TIME OF TREATMENT (MINUTES)	PERCENTAGE GERMINATION	AVERAGE NO. OF CELLS PRODUCED
10.....	91	2.8
20.....	91	2.6
30.....	90	2.5
40.....	89	2.6
50.....	90	2.7

It is apparent from table 1 that the germination and growth are uninfluenced by the calcium hypochlorite, at least up to a 50-minute treatment. These cultures were permitted to develop to the large prothallial stage. Observations at this later period revealed no differences between cultures of the various treatments.

SOWING SPORES.—For sowing the spores in the tube, a looped platinum needle such as is used in bacteriological work was employed. The spores float to the surface of the hypochlorite solution and it is necessary only to dip the loop of the needle in the mass of floating material. By practice, an approximately equivalent amount may be removed each time and spread over the surface of the agar slope. Approximately 4000 spores were sown in each tube.

CULTURAL CONDITIONS.—All cultures were placed under a cloth shade in the greenhouse. Two layers of cheesecloth were used on

the top of the shade chamber, which was 3 feet in height, and a single layer of cheesecloth was used for the sides. The maximum light intensity was 400 foot-candles. The temperature ranged from 65° to 85° F., the lower temperature prevailing at night. In the daytime effort was made to keep the temperature close to 75° F.

ISOLATION OF PROTHALLIA.—Ten to 12 days after sowing, the spore coat was ruptured and the first rhizoid made its appearance. Observations on germination and growth were begun on the twelfth day and made at intervals thereafter. At the very outset of the experiments some chloroplast differences were noted. Attempts to isolate protonema with abnormal plastids were made but such transplants failed to survive. Cultures nos. 18 and 188, however, were isolated when in young prothallial stages of thirty and fifty cells respectively. These will be described subsequently. Most of the prothallia exhibiting definite plastid abnormalities were isolated when in a more advanced stage.

All prothallia for examination were mounted in water on a microscope slide and gently teased apart with dissecting needles under a dissecting binocular microscope, using a magnification of 30×. Some of the plastid changes could be observed with this magnification although initially some of the prothallia with plastid abnormalities were missed. Later, prothallia were examined under a magnification of 90×. To isolate abnormal or other desired prothallia, use was made of flexible glass needles made by drawing out glass rods. These needles were about 20  $\mu$  in thickness and could be inserted under the coverglass and brought to the desired prothallium, which was pushed to the edge of the slide. It was then placed on a separate slide, and using the same glass needles the prothallium was freed of any adherent spores, protonema, or other prothallia. This prothallium was then picked up by means of a fine steel needle and transferred to a culture tube containing the nutrient medium.

REPRODUCTION OF PROTHALLIA.—After isolation, the prothallium of course no longer remained free of microorganisms. The tubes to which such isolated prothallia were transferred contained only the nutrient salts and no sugar. It was not therefore a medium favorable to the growth of microorganisms. Nevertheless some of the prothallia failed to survive the transfer. Some were bruised in the

handling and became covered later with a bacterial or fungus growth. This was especially the case when young prothallia (30- or 40-celled stage) were transferred. Fortunately prothallia representative of all types survived and reproduced vegetatively. It was observed early in the work that, under the greenhouse conditions provided, antheridia are not formed. Sexual reproduction of sporophytes is thus not possible and the prothallium continues to grow and reproduce new prothallia. At first protonemal threads arise from the prothallium, developing quickly into prothallia. Ultimately the entire agar surface may be covered with prothallia, which may be heaped up ten or more in depth. The quantity of prothallia obtainable is limited then only by the number of tubes or flasks provided.

#### Experimentation

Before considering plastid changes, the results of the effects of X rays on germination and growth will be presented. This is particularly desirable since there appears to be close correlation between plastid changes and the effect of X rays on germination and growth. For germination studies, samples were taken from representative cultures and observations made on at least 400 spores. The first observations were made at the end of 12 or 14 days, when the first evidences of germination may be noted. Subsequent observations were made at intervals until prothallia had reached an advanced stage. In the controls there was no appreciable increase in the percentage of germination after 20 days, but in those cultures sown with spores treated at 25,000 r units or more some germination occurred after this time, although no very significant increases were noted. Germination was considered to have occurred when the spore coat became ruptured. In those cultures with high intensities of treatment, 25,000 r units or more, many of the spore cells merely enlarged, their plastids developed, and such single-celled individuals would continue to live and enlarge, remaining in a healthy state. Observations on some of these single-celled individuals were made over a period of 18 months. How much longer they would have survived cannot be stated.

In addition to the data on germination, growth measurements were made at approximately the same intervals. These measure-

ments consisted merely in counting the number of cells of the protonema or young prothallia.

The data on germination are shown in figure 1. They are relative values and represent the results of three series of experiments, series 9, 10, and 11. In these the control is given a relative germination value of 100. The actual values for the controls of the three series were respectively 81, 75, and 96 per cent. The data were taken when germination of the control was practically complete.

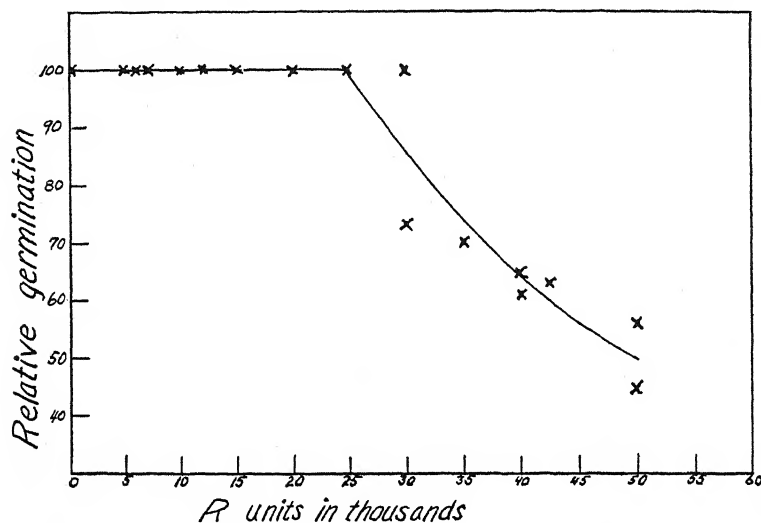


FIG. 1

In experiment 10, the treatments of spores were 0, 7000, 10,000, 12,000, 15,000, and 20,000 r units. Germination varied from 73 to 78 per cent, and to all these a relative germination value of 100 was assigned. This is in agreement with other experiments. In experiment no. 9 a series of treatments from 20,000 to 50,000 r units was provided by increments of 5000 r units. The percentage germination for the control was 81 and for the 30,000 r treatment 79. In experiment 11, however, the percentage germination in the culture with 30,000 r unit treatment was 77 against 98 for the control. The decrease in germination first occurs therefore at about 30,000 r units.

Under favorable conditions spore germination has occurred before the twelfth day and one cell division has occurred, and within a month a 12-celled protonema may be observed. Figure 2 gives the relative numbers of cells for the treatments 0 to 50,000 r units. These are taken from experiments 9 and 10 since the observations in these experiments were made at the end of approximately one month. The data from 20,000 to 50,000 r units are not only rela-

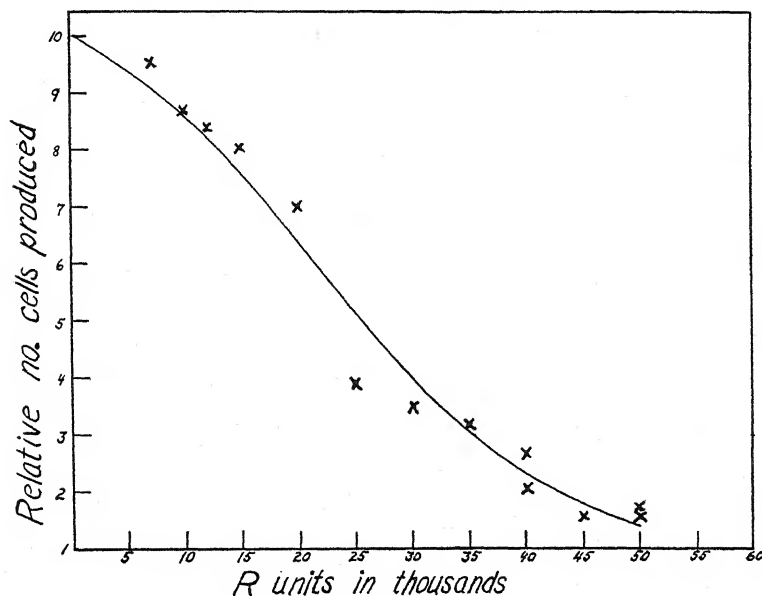


FIG. 2

tive but actual figures, since the control had made a growth of ten cells. The data for experiment 9 are given also in table 2. The data on growth reveal a progressive decrease with increasing intensities of irradiation. As shown by table 2 and also in figure 2, a large percentage of the plants show only a few cells. Nevertheless at these higher intensities some prothallia develop and attain a large size. It was among these that the isolations were made.

In experiments 9 and 11 a total of seventy-two cultures was provided. From twenty-five of these, fifty-one prothallia with abnormal plastids were isolated, the greatest number being obtained from ex-

periment 11 and particularly from series 11B. It is probable that in experiment 9 many prothallia with abnormal plastids were inadvertently missed. No abnormal plastids were observed in cultures treated with less than 30,000 r units. As indicative of the maximum frequency of these abnormal prothallia, consideration may be given to series 11B. In this series with sixteen cultures a total of approximately 64,000 spores were sown. From these approximately 12,672 prothallia developed from which thirty with abnormal plastids were

TABLE 2

EFFECT OF X RAYS ON GERMINATION AND GROWTH, EXPERIMENT NO. 9; DURATION OF EXPERIMENT, 30 DAYS

IRRADIATION	PERCENTAGE GERMINATION	AVERAGE NO. OF CELLS FORMED	PERCENTAGE 2-CELLED
Control.....	81	10.17	.....
20,000 r units.....	83	7.01	31
25,000.....	80	3.95	41
30,000.....	79	4.13	42*
35,000.....	57	3.19	48*
40,000.....	53	2.08	49*
45,000.....	51	1.62	84*
50,000.....	37	1.64	83*

\* Or less than 2-celled.

isolated. The ratio is therefore 1 to 422, or on the basis of total number of spores, 3 to 6400. In series 11C the ratio is 1 abnormal prothallium to 2050 normal, and in series 11D the ratio is 1 to 242. In series 11D the total number of prothallia produced was only 2176 but the number of spores sown in the sixteen tubes was again 64,000. When the frequency is based on the number of spores sown, the ratio of abnormal plastid forms becomes very low. In series 11D this ratio is about 1 to 7000. Data on isolations of prothallia with abnormal plastids are given in table 3.

These isolations of prothallia with abnormal plastids (table 3) were made in the latter part of 1932. Since that time they have been multiplied by vegetative reproduction. They have been grown on the usual culture medium in tubes, in liquid culture media, on soil, and on osmunda peat. Irrespective of the culture medium used, the characteristics of the plastids for each type have been main-

tained except for those slight variations which are associated with nutritional conditions. Except for the prothallia with grouped plastids, there is little difference among the prothallia having the different plastid types. These abnormal plastids have persisted then in the gametophyte for more than 7 years, and it may be concluded that the plastid change in this gametophyte generation is permanent and will persist as long as prothallia are reproduced vegetatively.

TABLE 3  
NUMBER OF PROTHALLIA SHOWING ABNORMAL PLASTIDS  
ISOLATED FROM THE DIFFERENT EXPERIMENTS

SERIES NO.	R UNITS	NO. OF CULTURES	NO. OF ISOLATIONS	PERCENTAGE OF SPORES WHICH PRODUCED PROTHALLIA
11B.....	30,000	16	30	19.8
11C.....	40,000	16	4	12.9
11D.....	50,000	16	9	3.4
9E.....	35,000	6	1	25
9F.....	40,000	6	5	25
9G.....	45,000	6	1	8
9H.....	50,000	6	1	6

In order to simplify the presentation of data, a classification of the prothallia based on the plastids is presented in table 4. Including the normal type, eleven groups have been established. Within a single group there may be constant differences and therefore distinctive types, but the general similarity justifies the grouping and simplifies the discussion. Consideration has been given to the plastids in cells of all ages, but in the main the classification is based on the plastids in cells of intermediate age. In the very young cells the plastids are generally small and undeveloped; in the very old cells they may be extraordinarily modified. The distinctive characteristics of each are shown in the accompanying illustrations.

TYPE A, NORMAL PLASTIDS.—In prothallia produced from spores not irradiated, and likewise in nearly all prothallia produced from irradiated spores, the plastids are characteristically uniform in size and shape. They may be small, and platelike or discoid, with a thickness of about  $1.5 \mu$ . In surface view the plastids when crowded

TABLE 4  
CLASSIFICATION OF PROTHALLIA BASED ON PLASTID

TYPE	DESCRIPTION OF PLASTIDS	CULTURE NO.	ORIGINAL EXPERIMENT	IRRADIATION (R UNITS)
A.....	Normal; small, roundish, or angular plates, uniform in shape	.....	.....	None
B.....	Grouped; collected in mass about nucleus	{ 96 121 187 188 195 272	9F3 11D5 11B8 11B8 11B5 11B6	40,000 ..... 30,000 30,000 30,000 30,000
C.....	Giant; amoeboid forms; frequently vacuolate	{ 18 198	11C9 11B4*	40,000 30,000
D.....	Large thin plates; incomplete divisions or fusion with plastids stringlike, branched; others irregular; some vacuolate	{ 91 291	11C16 11B6*	40,000 30,000
E.....	Very large plates; in older cells slightly more irregular	262	11B7*	30,000
F.....	Large thin plates appearing as mosaic; in older cells very irregular with fusions	263	11B7	30,000
G.....	Large thin plates, irregular outline, incomplete division or fusions; extraordinary vacuolations	185	9F4	35,000
H.....	Large plastids; square, rectangular, or rounded plates	{ 79 85 206 231 233 249 250 255 266 267 282 296	11B10 ..... 11B9 ..... 11B4 11D9 11D9 11D9 ..... ..... 11B6 11B6	30,000 ..... 30,000 30,000 30,000 50,000 50,000 ..... ..... 30,000 30,000
I.....	Thin plates, variable in size, not large; surface appears rough owing to many starch grains	148	.....	.....
J.....	Small plastids; roundish to angular, others irregular, some vacuolate; relatively large starch grains; tending to group	235	.....	.....
K.....	In young cells plastids small, rounded; in older cells free of starch; spherical, with chlorophyll in bands or threads; vacuolate	205	.....	.....

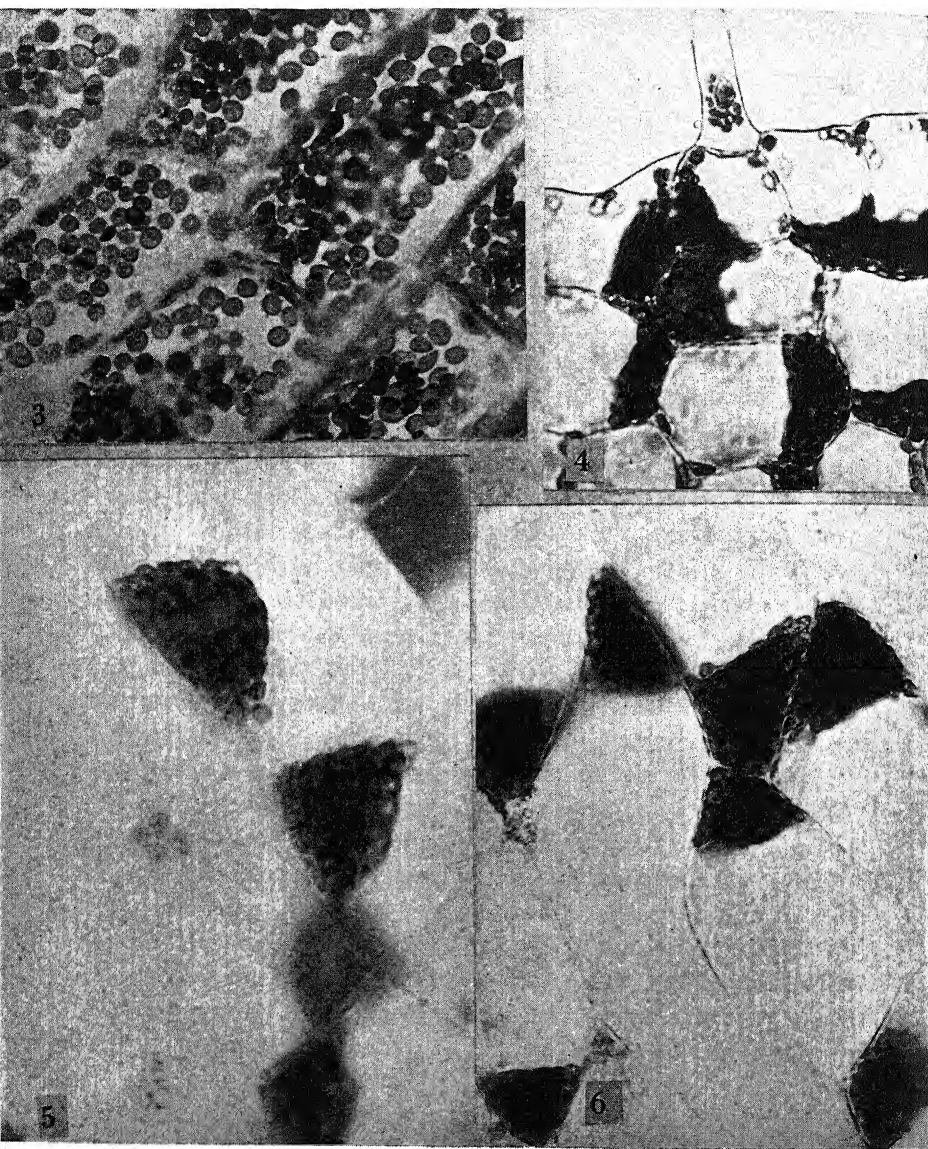
\* Grown with 2% sucrose.

are angular, being squarish or rectangular to polyhedral; when less crowded they may be rounded in outline. In surface view they vary in size from  $3 \times 4.5 \mu$  to  $6 \times 9 \mu$ . These values hold for plastids in healthy cells; in poorly nourished cells they may be appreciably smaller. Another characteristic is the large number of plastids per cell. When the plastids are uniformly distributed, 125 or more may be noted in either the upper or the lower periphery of the cell (fig. 3).

TYPE B, GROUPED PLASTIDS.—In the prothallia of *Polypodium aureum* and in plants in general the chloroplasts are distributed in the peripheral cytoplasm. Preceding cell division there may be a clustering of the plastids about the nucleus, but this grouping does not persist. In epidermal cells leucoplasts may be clustered about the nucleus, as in *Zebrina pendula*, and in *Elodea* under high light intensity such a clustering may occur. In the prothallia of group B the plastids are massed about the nucleus, generally near an end wall. Only occasionally is a plastid or a group of three or four found isolated in the peripheral cytoplasm (figs. 4-6). In these prothallia the nucleus of a cell is not visible and its position is made manifest only when light of high intensity is used. Under such conditions its position is made evident by a light spot within the mass of plastids. The extent of the grouping varies within the members of this group. Thus in cultures nos. 96, 272, and 195 it is rare to find an isolated plastid. In nos. 187 and 188 small groups of plastids may be isolated in the peripheral cytoplasm. It is difficult to measure the plastids grouped about the nucleus, since many show only an edge view while others appear to be distorted in shape. Those that are isolated are roundish plates. In no. 96 the diameter ranges from  $4.5$  to  $7.5 \mu$ , in no. 121 from  $3$  to  $7.5 \mu$ , in nos. 87 and 88 from  $4.5$  to  $7.5 \mu$ , and in nos. 195 and 272 from  $4.5$  to  $6 \mu$ . In size these plastids are like the normal.

These prothallia are thin-walled and are bluish green in color, although no. 272 is slightly more greenish than others of this group. The prothallia are easily distinguished macroscopically from all other types because of their unusual color and more delicate structure. They also appear to grow more slowly.

Light intensity is not a factor of importance in this grouping.



FIGS. 3-6.\*—Fig. 3, type A: Typical normal plastids in prothallia of *Polypodium aureum*; note small size and large number in surface view. Figs. 4-6, type B: Grouped plastids densely packed about nucleus (fig. 4, culture 121; fig. 5, culture 188; fig. 6, culture 96); in figs. 4 and 5 a few plastids may be noted isolated in peripheral cytoplasm.

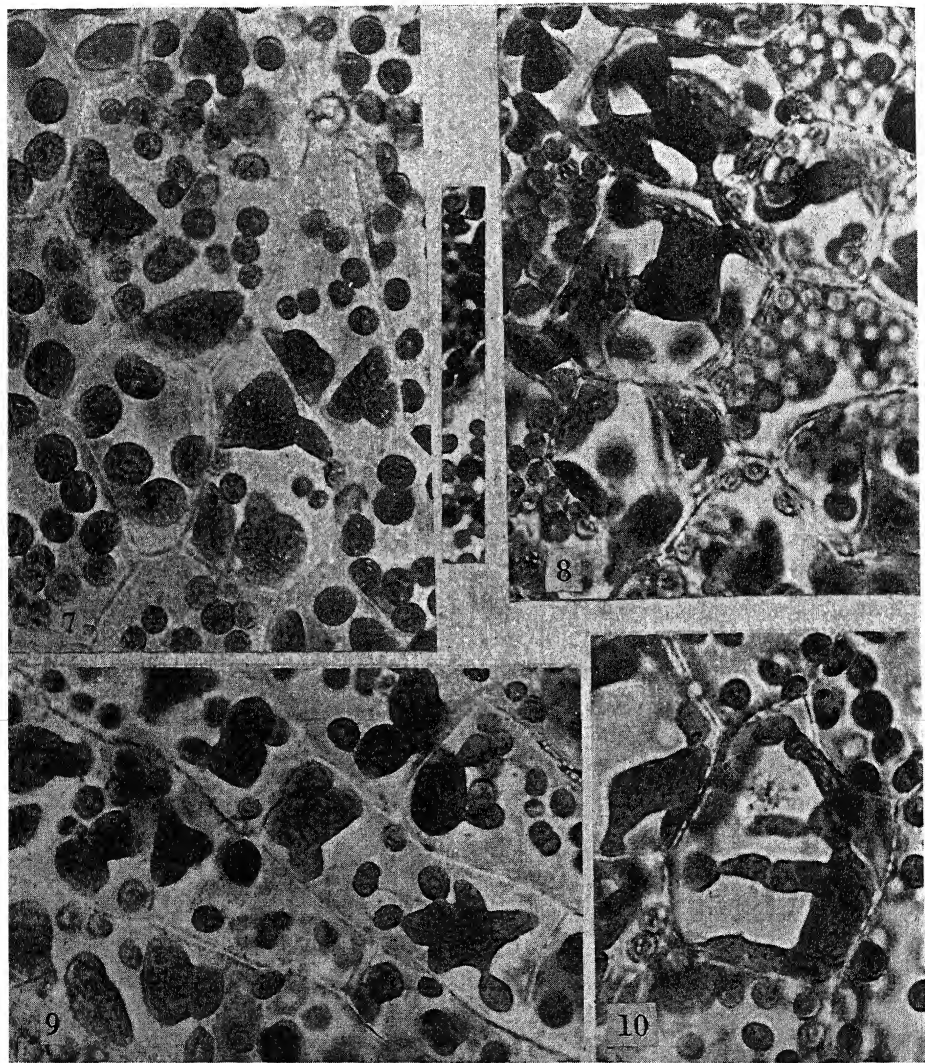
\* Magnification in all figures is 500.

Cultures kept for 5 months under a laboratory bench, where the light intensity during the daytime was only 5 foot-candles, still exhibited plastids grouped about the nucleus. In the greenhouse, in cultures maintained under conditions where light intensity reached values of 2000 foot-candles, the grouping persisted.

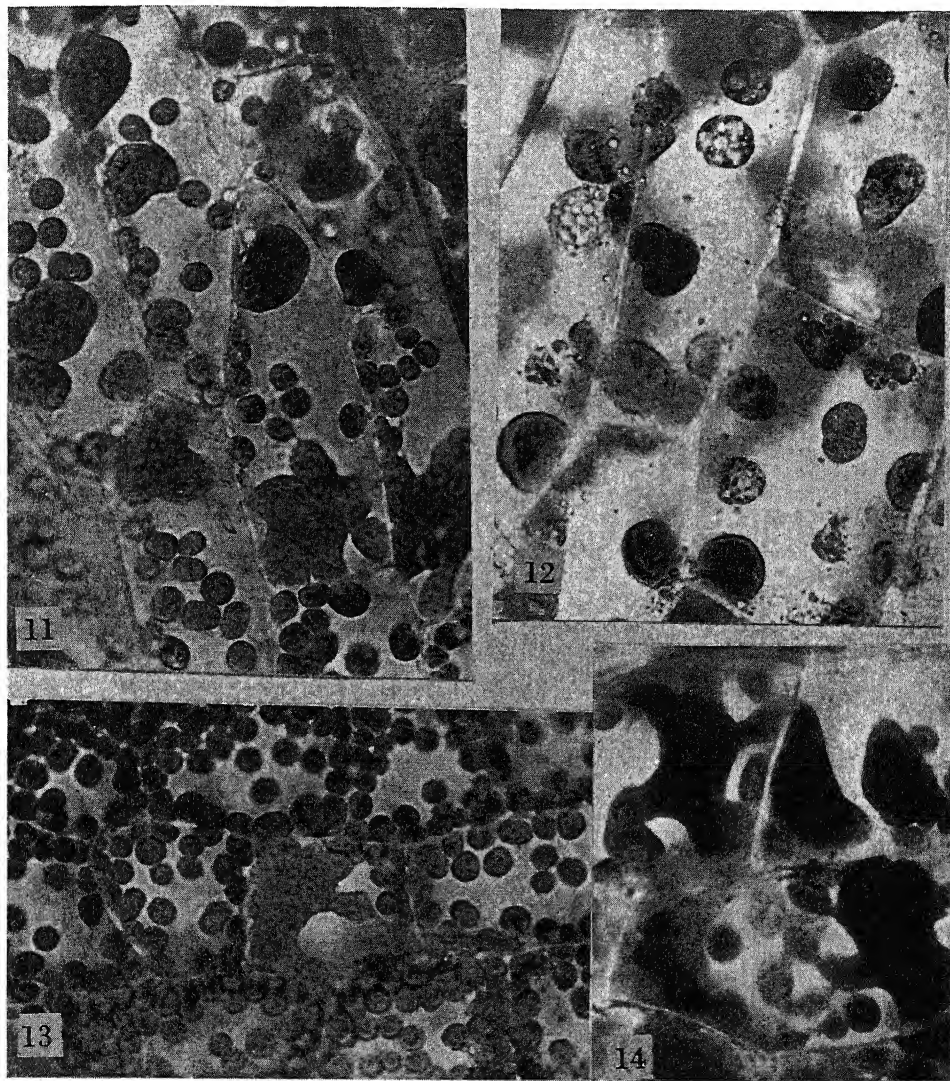
TYPE C, GIANT PLASTIDS AND AMOEBOID FORMS.—Only two prothallia of this type were isolated, nos. 18 and 198. The group is characterized by very large and irregularly shaped plastids located mostly in a layer of five-ten cells bordering the periphery of the prothallium. Because of the shape of these plastids they are termed amoeboid. They may be present in any cell of the prothallium except the apical and those younger cells bordering the notch. In these young cells the plastids are circular in outline and only  $2-3\ \mu$  in diameter. There are about ten of these small plastids in each of the very young cells. In the cells toward the base of the prothallium the plastids are generally circular plates, varying in healthy prothallia from  $7.5$  to  $15\ \mu$  in diameter. Even in these cells an amoeboid plastid may be found. The striking features of these prothallia are the amoeboid plastids found in the cells of intermediate age. Usually these plastids are more common on the lower surface of the cell than on the upper (figs. 7-11).

The typically amoeboid plastids vary greatly in size and shape and assume grotesque forms. The evidence appears to show that they are formed by the union of smaller plastids, and then by a budding process give rise again to the smaller plastids (figs. 7, 11, 14). Because of their curious outline it is difficult to measure them. In some cells a single large rectangular plastid may be observed with dimensions of  $50 \times 70\ \mu$ . Long bandlike plastids have been observed  $150 \times 6\ \mu$ . L-shaped plastids occur with the long arm  $60 \times 23\ \mu$  and the short arm  $45 \times 15\ \mu$ . Of the irregularly shaped plastids the maximum dimension may vary from 20 to  $60\ \mu$ . The frequency of the amoeboid plastids seems to be correlated positively with the vigor of growth of the prothallium.

Culture no. 198 is similar to no. 18 but appears to differ from it in two respects. The plastids tend to be more commonly circular in outline, with diameters of  $6-15\ \mu$ . The amoeboid forms are less frequent in no. 198 than in no. 18, but more plastids are "vacuo-



FIGS. 7-10.—Type C: Amoeboid type plastids, culture 18, illustrating striking size and varied forms. Figs. 7, 8, certain cells shown possessing only circular form of plastid. Fig. 9, yeastlike budding plastids shown clearly. Fig. 10, very large and irregular plastid. Insert, normal plastids.

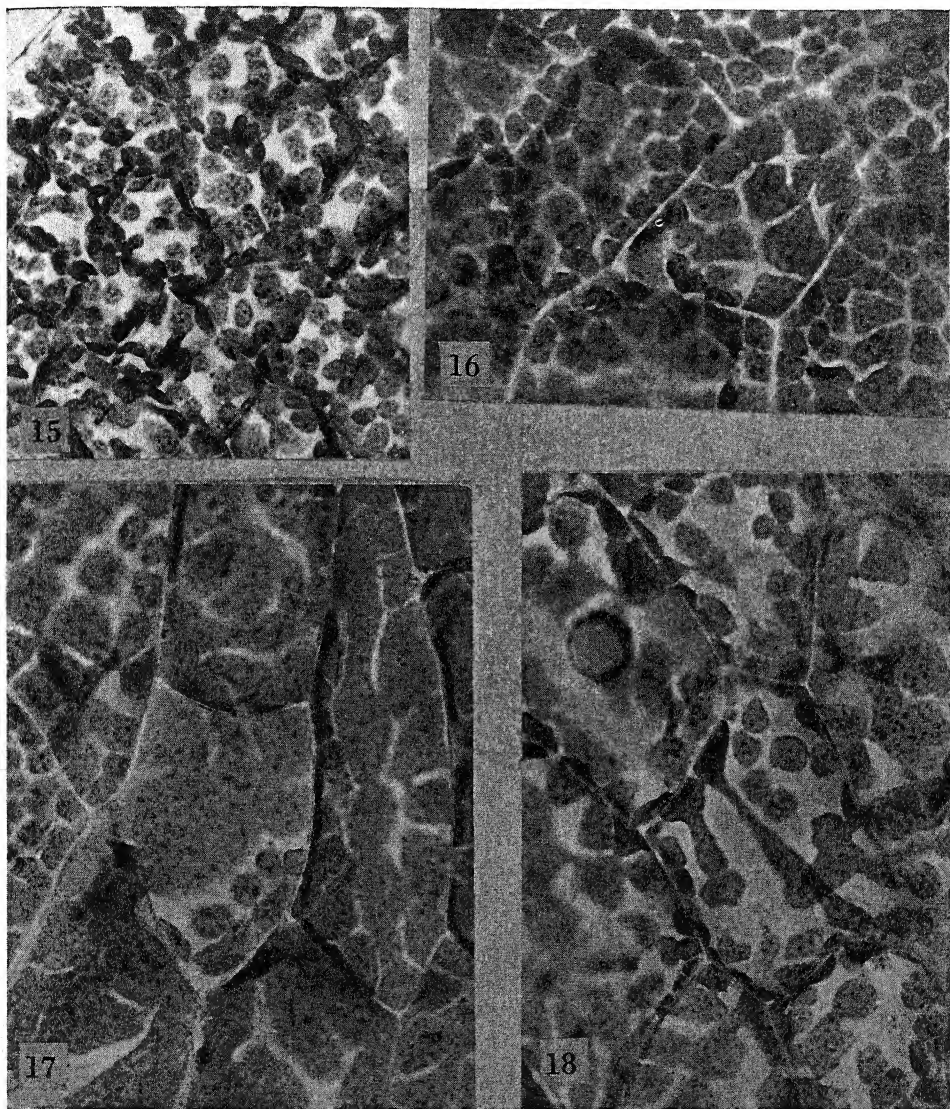


FIGS. 11-14.—Fig. 11 (type C, culture 18), showing yeastlike budding of plastids. Figs. 12-14, type C: Amoeboid plastids, culture 198. Fig. 13, inner cells of prothallium showing predominance of circular plastids; fig. 14, peripheral cells showing large amoeboid types; fig. 12, older cells showing vacuolate type plastids. Some plastids are swollen and chlorophyll is irregularly disposed, having the appearance of a foamlike structure owing to unusual disposition of chlorophyll.

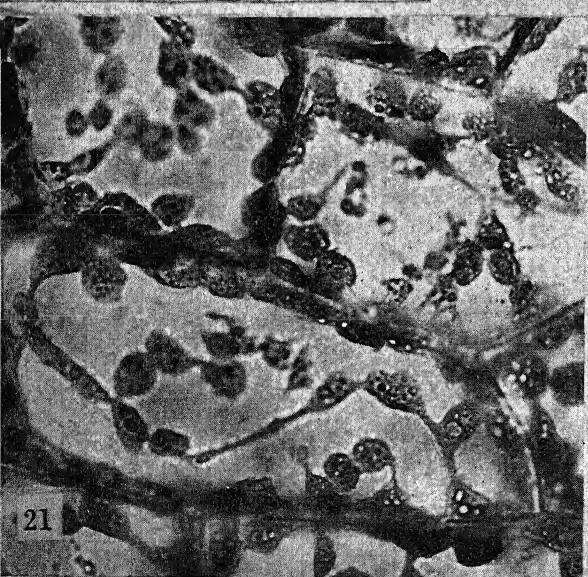
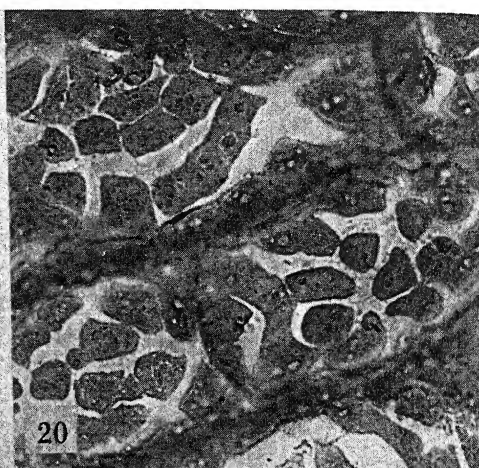
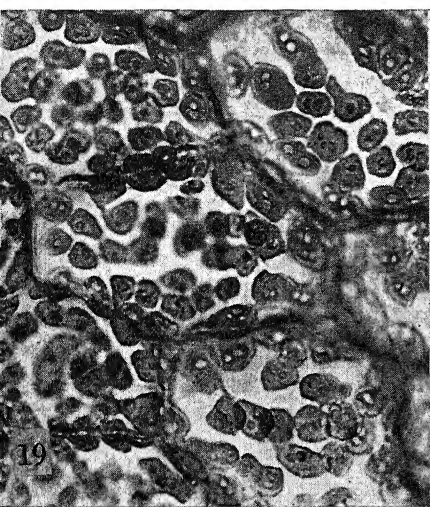
lated" in no. 198 than in no. 18 (fig. 12). Some of these vacuolated plastids present the appearance of rings with jewel settings. Others in outline form present the appearance of the figure 8. In reality the latter consists of two spheres and the ringlike plastids are nearly spherical in shape; in both cases the chlorophyll is not uniformly distributed but occurs in bands. This will be considered subsequently when discussing the plastids of group J.

TYPE D, LARGE THIN PLATES, IRREGULAR IN SHAPE, FUSIONS.—Cultures nos. 91 and 291 have been placed in this group because in the very young and medium aged cells the plastids are much alike. In the young cells the plastids are angular plates of variable sizes; in the medium aged and older cells large platelike plastids prevail and are variable in size. These have dimensions of  $30 \times 3 \mu$ ,  $9 \times 9 \mu$ ,  $6 \times 3 \mu$ ,  $15 \times 16 \mu$ ,  $12 \times 9 \mu$ ,  $15 \times 12 \mu$ , and some with maximum dimension of  $60 \mu$  (figs. 15–18, 19–22). In cells of medium age, plastid masses may be held together by thin green threads. In the older cells the plastids seem to have fused, especially in no. 291 (figs. 21, 22). Vacuolated plastids are common (figs. 18, 22). This condition is so pronounced in the older cells that some plastids appear to have a frothlike structure (fig. 22). The starch grains in all plastids are large.

TYPE E, VERY LARGE THIN PLATES; CULTURE NO. 262.—Only one prothallium of this type was observed and isolated. It survived and reproduced other prothallia in abundance. It is a vigorous grower. In the very young cells adjacent to the apical cell the plastids tend to be rodlike, expanding in size to small plates in older cells. In fully formed cells of intermediate age—and therefore in the greater part of the prothallium—the plastids are thin plates and attain extraordinary size. In outline they are generally angular and various sizes may occur in the same cell. The smaller plastids vary from  $10 \times 8 \mu$  to  $20 \times 6 \mu$ . The large ones may be  $60 \times 38 \mu$ ,  $20 \times 20 \mu$ , or  $45 \times 22 \mu$  (fig. 23). In the older cells (figs. 24, 25) the plastids are generally smaller, being strandlike with swollen ends, recurved, circular in outline, or bands. In all of them the starch grains are numerous and very prominent. These plastids, while possessing a large area, are usually thinner than are normal ones.



FIGS. 15-18.—Type D: Irregular thin plates, culture 91. Fig. 15, young cells near growing point; plastids irregularly shaped plates and relatively large for such cells. Figs. 16, 17, later stages in plastid development. Fusion appears to have occurred, yielding large plastids as in fig. 17. Fig. 18, irregularity of shape and vacuolation of plastid.



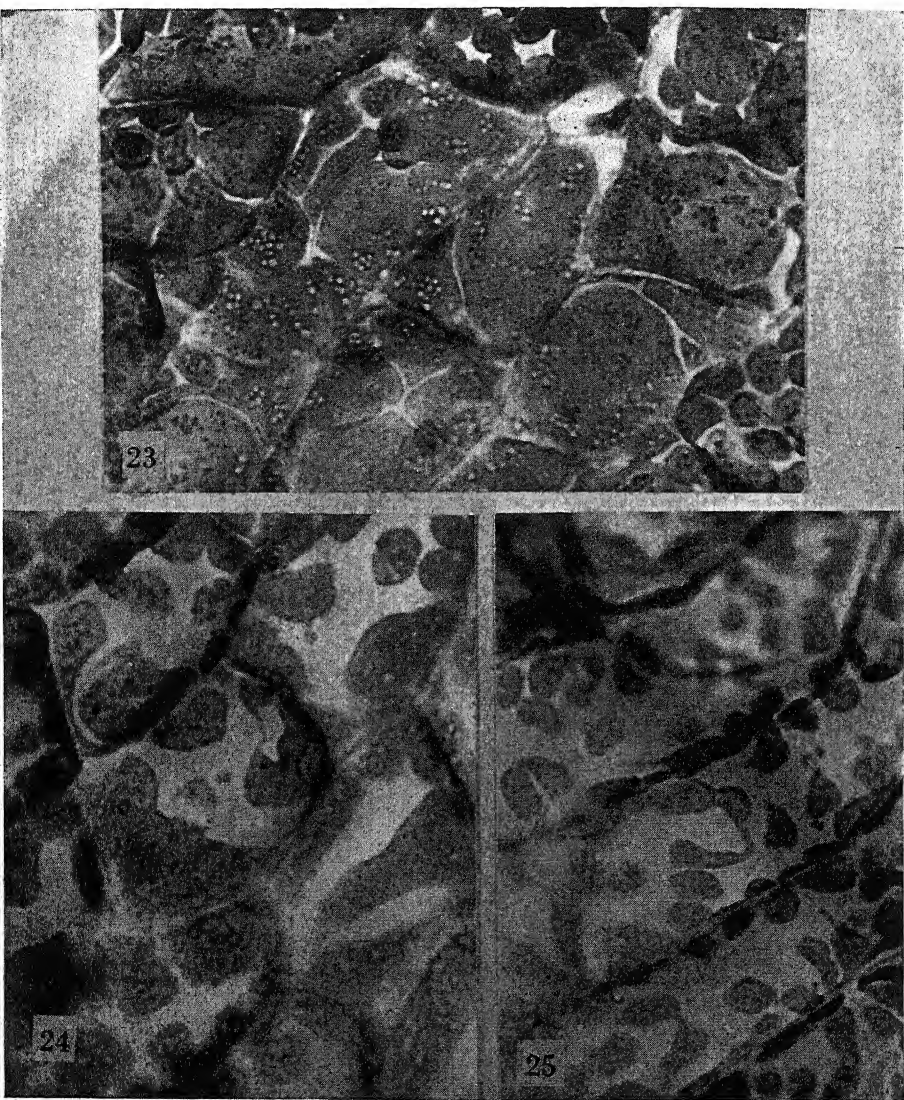
FIGS. 19-22.—Type D: Culture 291. Fig. 19, young cells. Fig. 20, older cells showing irregularly shaped plate plastids. Fig. 21, fusion of plastids. Fig. 22, vacuolization.

GROUP F, IRREGULAR THIN PLATES, FUSIONS; CULTURE NO. 263.—In the very small cells close to the apical cells the plastids are similar to those of no. 262, appearing as small rods or angular or circular plates. In cells slightly older many of the plastids appear to fail to separate, remaining connected by thin green strands (fig. 26). In the older cells the plastids, while platelike, show great irregularity in shape and size. In these older cells of healthy prothallia, this irregularity of outline is suggestive of the irregular splitting of a thin film of clay and may be likened to the appearance of a jigsaw puzzle (fig. 27). In the large mature cells the plastids become elongated masses of plastid material connected by long green strands (fig. 28). In many of these older cells fusions appear to have occurred, with most of the plastids interconnected by green strands (fig. 29) in which small starch grains may be noted.

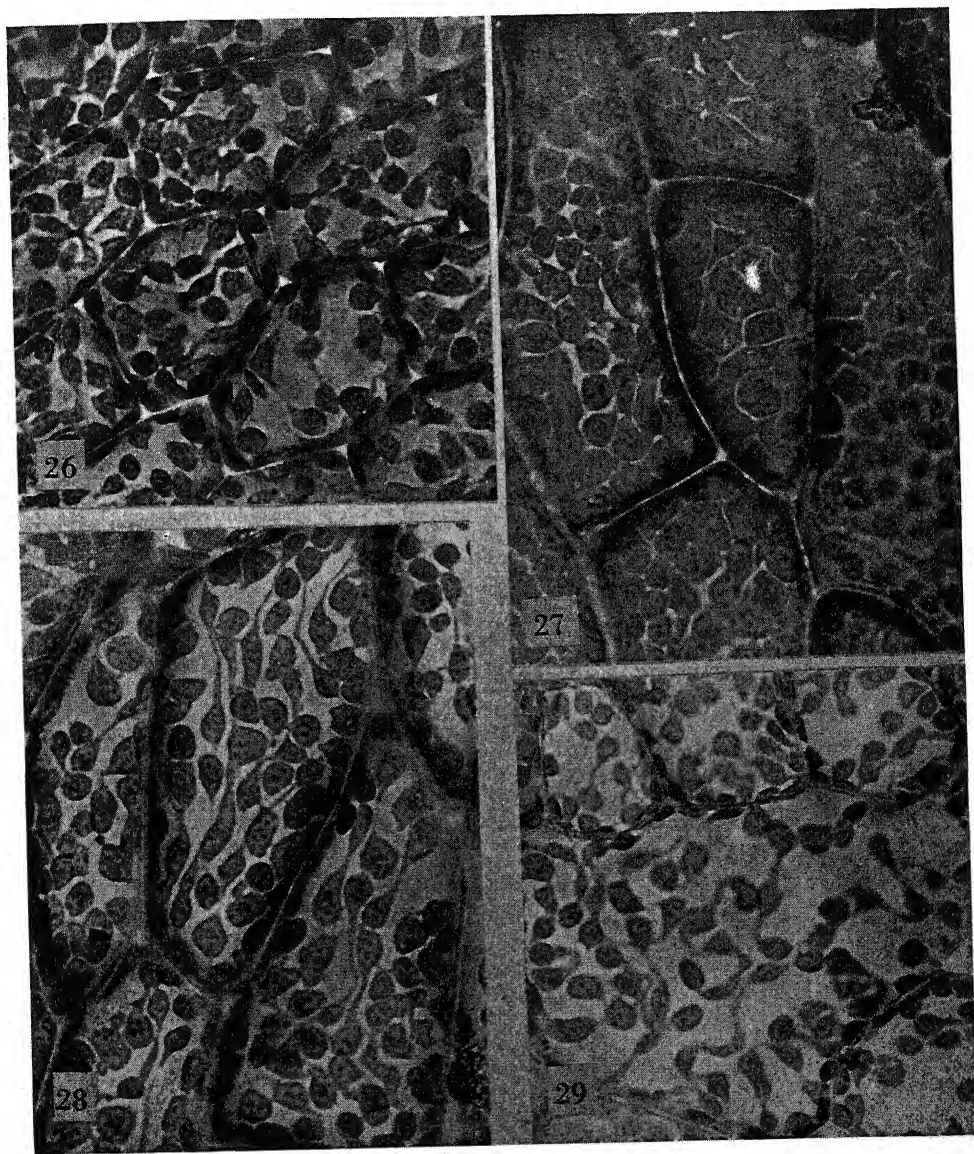
In general the plastids of no. 263 are smaller than those of no. 262 and more numerous per cell. Because of the irregularity of shape it is difficult to measure the plastid. In cells of intermediate age those plastids that can be easily measured show dimensions as follows:  $9 \times 9 \mu$ ,  $7 \times 4 \mu$ ,  $5 \times 15 \mu$ ,  $21 \times 9 \mu$ ,  $25 \times 15 \mu$ ,  $33 \times 8 \mu$ , and  $30 \times 12 \mu$ . The largest rectangular plastid measured was  $90 \times 18 \mu$ .

TYPE G, THIN PLATES, VARIABLE IN SIZE, VACUOLATED; CULTURE NO. 185.—Only one prothallium of this type was isolated. In the apical and adjacent cells the plastids are minute, and angular or circular in outline. These quickly enlarge, so that in young cells platelike plastids appear with prominent starch grains (figs. 30, 32). In cells slightly older, larger plates are noted. These are irregular in outline and many of the plastids are vacuolated (fig. 31). Plastids with enlarged ends connected by thin green strands are common (fig. 33). In old cells irregular bandlike plastids occur which appear to be the result of fusion. In cells of intermediate age the rectangular plastids have dimensions of  $6 \times 30 \mu$ ,  $6 \times 18 \mu$ , and  $9 \times 18 \mu$ . Circular plates  $15 \mu$  and some only  $7 \mu$  in diameter may be observed.

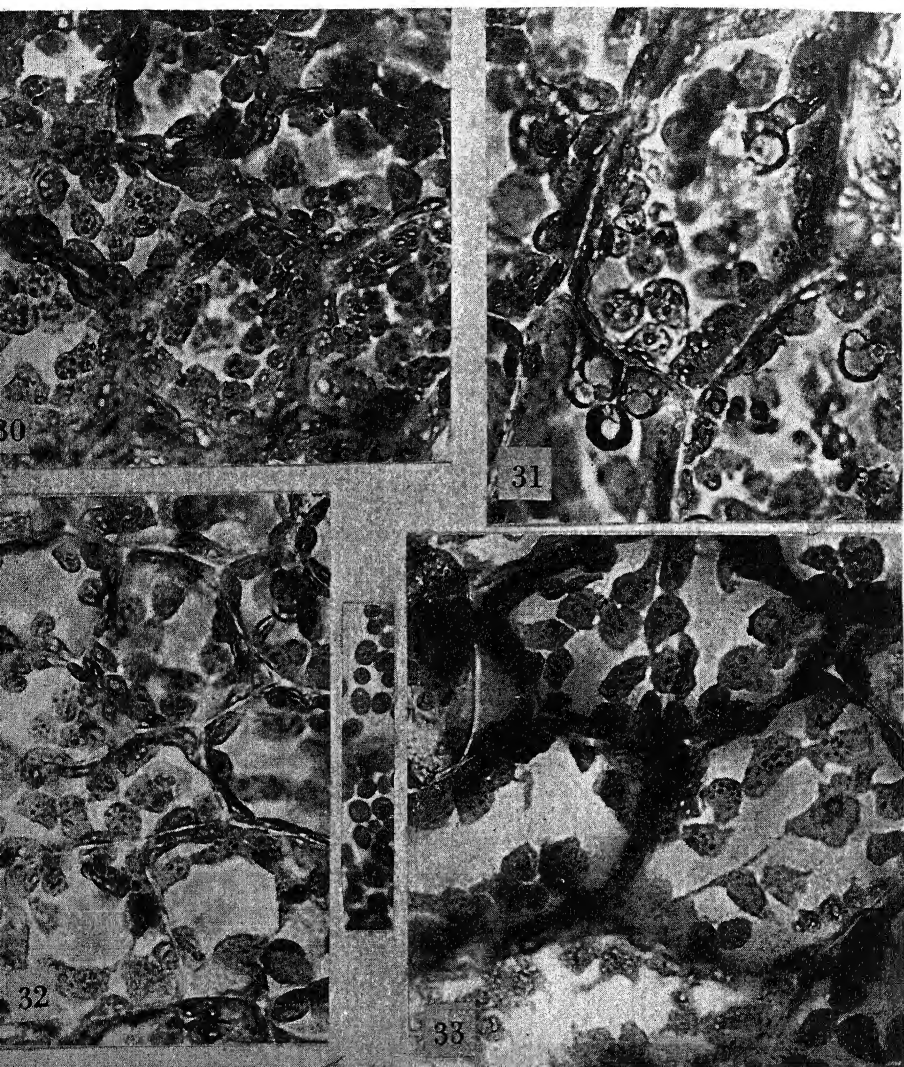
TYPE H, LARGE PLASTIDS.—This type of plastid change occurred with the greatest frequency. Altogether twenty-one prothallia exhibiting large plastids were isolated, and of these twelve survived. For the most part they are much like the normal ones in shape and



FIGS. 23-25.—Type E: Culture 262. Fig. 23, relatively young cells with very large plate plastids containing many starch grains, and smaller plastids interspersed. Fig. 24, shrinkage of plastids in older cell. Fig. 25, older cells; plastids relatively small plates and strandlike.



FIGS. 26-29.—Type F: Irregular plates and fusion, culture 263. Fig. 26, young cells near growing point; strandlike connections between plastids. Fig. 27, older cells; plastids forming pattern like jigsaw puzzle, characteristic of type F. Fig. 28, long green strands connecting enlarged portions of plastid. Fig. 29, fusion resulting in long meandering plastid.



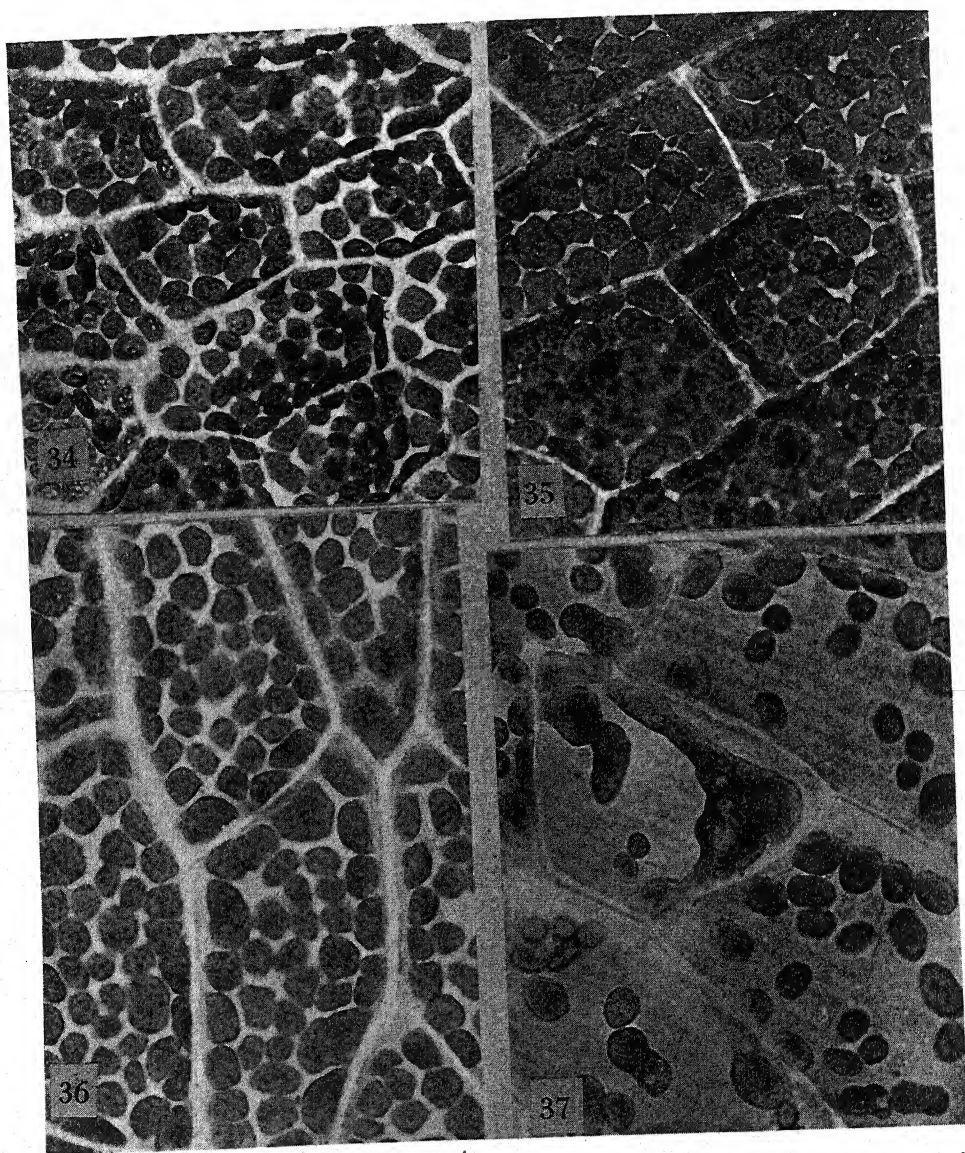
FIGS. 30-33.—Type G: Culture 185. Figs. 30, 32, young cells showing irregularity of size and shape plastids. Fig. 31, older cells with larger plastids, some vacuolate. Fig. 33, medium aged cells with plastids possessing thin strands connecting swollen ends of plastid. Insert, normal plastids.

form but are very much larger, having at least twice as much area (figs. 34-37). Culture 296 exhibits at times unusually large plastids somewhat similar to the amoeboid type, but for the most part the plastids conform to the type of this group. In young cells (fig. 34) the plastids are angular plates 6-10  $\mu$  in maximum dimension. In older cells they are angular plates, although with fewer plastids to the cell circular plates may prevail. These tend to be variable in size. The circular plate types have diameters of 9-21  $\mu$ ; the rectangular plastids vary from 6  $\times$  15  $\mu$  to 15  $\times$  22  $\mu$ . Some tend to become vacuolated, although this is not common. In general the number per cell is much less than in the normal. In the normal cell there may be 125 or more in a surface view, but only thirty to forty of the large plastids are present in the peripheral cytoplasm.

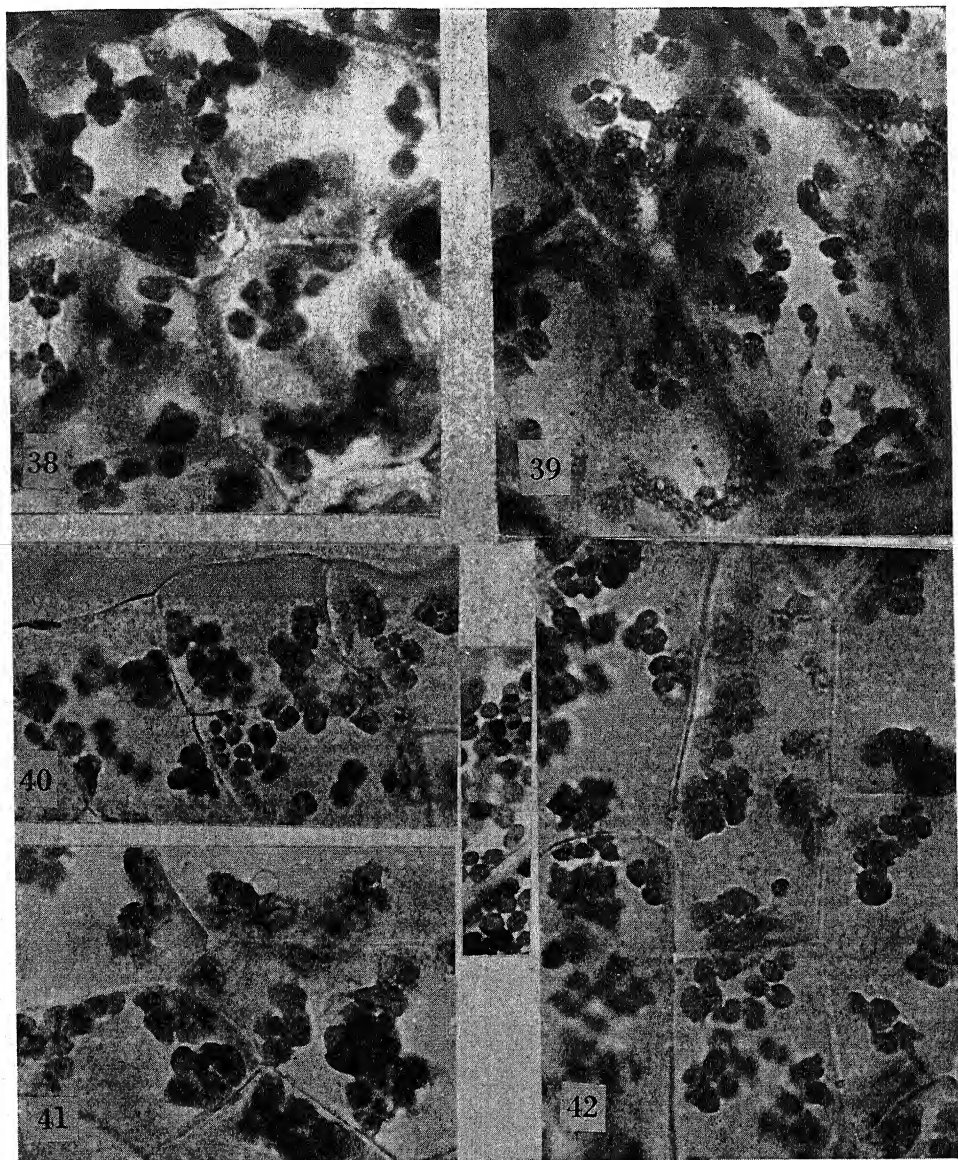
TYPE I, THIN PLATE GENERALLY SMALL, VARIABLE IN SIZE; CULTURE NO. 148.—Only one prothallium of this type was isolated. In the very young cells the plastids are minute plates, circular or angular in outline. In well developed cells they are thin plates with dimensions of from 3  $\times$  4.5  $\mu$  to 7.5  $\times$  7.5  $\mu$ . This is about the size of the normal plastids but larger ones may also be noted, some having dimensions up to 15  $\times$  18  $\mu$ . Some are connected by thin green strands, while vacuolated plastids are not uncommon. The surface of the plastid appears rough because of the large starch grains (figs. 38, 39).

TYPE J, PLASTIDS SMALL, VARIABLE IN SHAPE, AND TENDING TO GROUP; VACUOLATED; CULTURE NO. 235.—These plastids resemble those of culture no. 148 of type I, but they are more uniform in size, smaller, and tend to group. The circular plate plastids may vary in size, diameters being from 4 to 10  $\mu$ . The rectangular plastids have dimensions of from 4.5  $\times$  6  $\mu$  to 6  $\times$  7  $\mu$  (figs. 40-42). A few large ones may have dimensions of 4.5  $\times$  12  $\mu$ . Strandlike plastids are occasionally noted. Vacuolated plastids occur and in nearly every cell in the plane of the plastids a colorless ringlike structure may be observed. Such rings are variable in size (figs. 41, 42) and are probably plastids free of starch and chlorophyll which have assumed the vacuolated condition.

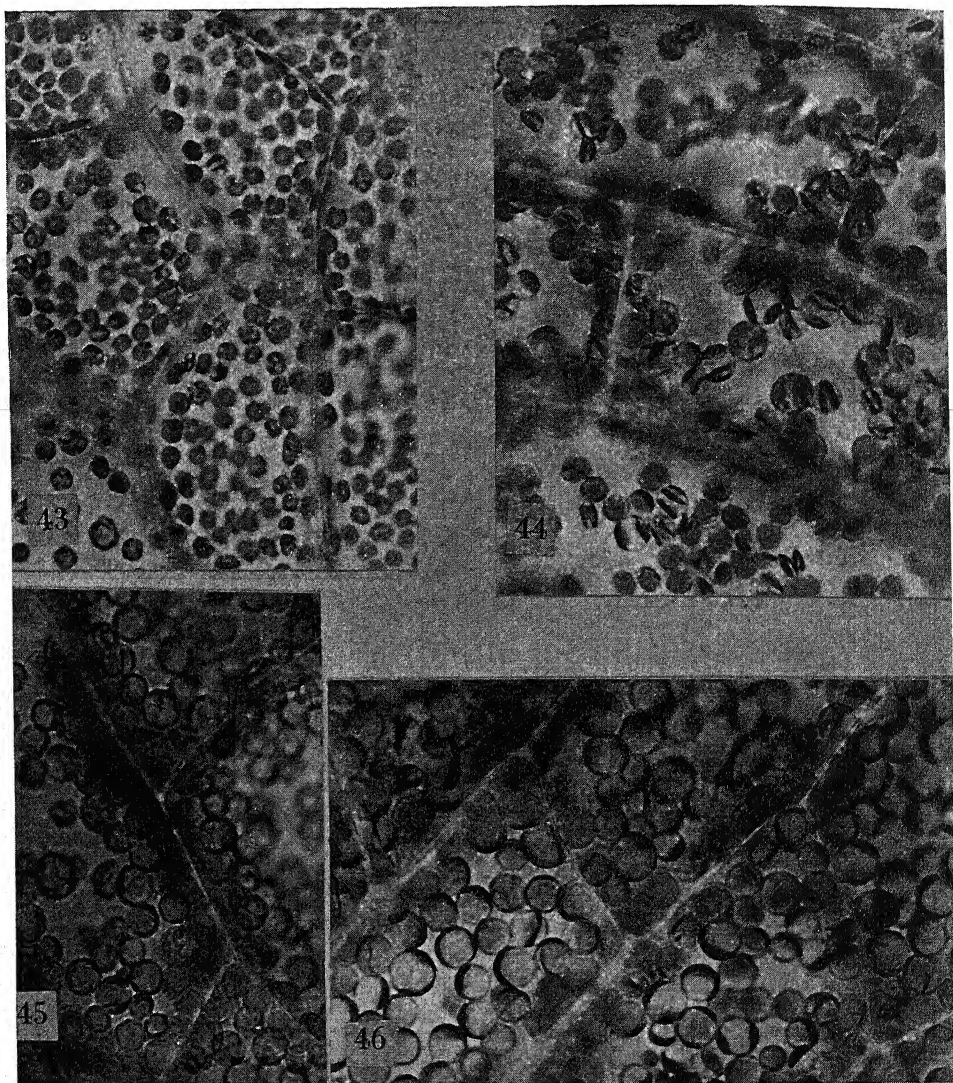
GROUP K, VACUOLATED PLASTIDS; CULTURE NO. 205.—Two prothallia of this type were isolated but only one survived. This is



FIGS. 34-37.—Type H: Large plastids. Fig. 34, young cells near growing point of prothallium, culture 266; note small number of plastids and relatively large size. Fig. 35, slightly older cells of same prothallium. Fig. 36, mature cell of culture 266, showing characteristic large plastids. Fig. 37, unusual plastid found occasionally in culture 266.



FIGS. 38-42.—Figs. 38, 39, type I: Culture 148. Fig. 38, plastids in young cells; fig. 39, old cells with plastids tending to coalesce. Figs. 40-42, type J: Culture 235. Fig. 40, young cells near growing point; figs. 41, 42, older cells showing grouping tendency; ringlike structures, probably vacuolate plastids, shown especially well in figs. 41 and 42. Insert, normal plastids.



FIGS. 43-46.—Type K: Culture 205, vacuolate type. Fig. 43, young cell showing typically small plastids with chlorophyll concentrated in small bands and threads. Fig. 44, slightly older cells with chlorophyll in band and threadlike arrangement. Starch entirely lacking in these plastids and in those of figs. 45 and 46. Fig. 45, old cells showing plastids with equatorial bands of chlorophyll, others with bands and irregular threadlike strands of chlorophyll. Fig. 46, mature cells; chlorophyll largely disposed in equatorial band.

probably the most extraordinary plastid of all. In young cells of healthy dark green prothallia the plastids appear almost normal, except that their appearance suggests a slightly more liquid consistency. Starch is present in small granules and is scant. In slightly older cells the plastids show an irregular distribution of chlorophyll, possessing islets free of chlorophyll (fig. 43). In slightly older cells the plastid is entirely free of starch and appears spherical, with the chlorophyll in wide bands and thinner interconnecting bands. In still others the chlorophyll appears as an equatorial band. Many such variations occur (figs. 44-46). This type grows extremely slowly and is difficult to maintain in culture.

In seeking an explanation of this unusual distribution of chlorophyll, consideration must be given to the osmotic properties of the plastid. PRIESTLEY and IRVING (22) pointed out that plastids of a species of *Selaginella* when extruded into distilled water showed marked swelling and finally rupture. This swelling was likewise observed by LIEBALDT (17). PRIESTLEY and IRVING considered the swelling to be due to colloidal imbibition. In a brief paper I (13) emphasized the osmotic properties of the plastid and pointed out the probable presence of a semipermeable membrane. This will be reported in detail subsequently. Suffice it to state here that these plastids exhibit certain characteristics of plastids extruded into distilled water. They are spherical in shape and apparently the plastid material has actually pulled away from the chlorophyll in expansion. When a cell containing these plastids is plasmolyzed, these abnormal plastids are changed back to a type approaching the normal.

#### Discussion

It is not the purpose of this paper to consider primarily the physiological effects of X rays. The data on germination and growth, however, are the result of many measurements and warrant a few statements. In her summary of the effect of X rays on green plants, JOHNSON (10) properly points out that many investigators have used an inadequate number of plants or have had too few replications to warrant definite conclusions. As has been done by others, she points out that in general the effect of X rays is deleterious to plants. My data are in agreement with this viewpoint. The spores

of *Polypodium aureum* require a high dosage of X rays to influence the germination and growth. The curves on germination and dosage (figs. 1, 2) reveal that the percentage of germination is decreased only when the dosage is as high as 25,000 or 30,000 r units. The growth curve resembles the survival curve as reported by PACKARD (21) for *Drosophila*. There is no stimulation of growth as was reported by SHULL and MITCHELL (24) for certain higher plants. In other experiments I have used dosages of 500-50,000 r units and no stimulation was noted. It is true that SHULL and MITCHELL used filtrated radiation, but in view of PACKARD's work it is doubtful whether stimulation would result from filtered radiation. That the growth rate of *Polypodium aureum* can be used as a biological dosimeter is suggested by the results obtained, but at best the gametophyte stage of this plant could be used only to measure high dosages.

An interesting observation was the frequent occurrence of spores which failed to divide but continued to enlarge until large spherical cells were produced with diameters of approximately 100  $\mu$ . These cells were well provided with chloroplasts and contained large reserves of fat, usually with one large centrally disposed globule of fat surrounded by smaller droplets. Some of these cells appeared to be healthy after 18 months. How long they would have lived cannot be stated.

Mention has been made previously of the fact that fifty-one prothallia with abnormal chloroplasts were isolated. These were obtained only from cultures irradiated at 30,000 or more r units. Seventy-two such cultures were provided, each with about 4000 spores, so that to obtain these prothallia approximately 300,000 spores were sown. The type with large plastids yielded the greatest number of isolations and was followed closely by the type with grouped plastids. Thus of the fifty-one prothallia isolated, twenty-one had large plastids (type H), fourteen were of the grouped type (type B), five of the vacuolate type (type K), two of the amoeboid type (type C), and four of type D. Only one prothallium was obtained of each of the other types.

It has been emphasized that these new plastid types have persisted in culture for more than 6 years and that each type maintains its characteristics, irrespective of cultural conditions. Since

no plastid changes were found in cultures irradiated below 30,000 r units, it is clear that the changes were caused by irradiation. In other investigations in this laboratory with the same fern it is probable that several million prothallia have been examined microscopically. No plastid changes were observed.

In investigations on the effect of X rays on green plants, chlorophyll variations have been noted (18, 4), suggesting that these might involve structural changes in the plastids. The only reference to such changes are those which I (12) reported in 1934 and which are amplified in this paper, and the plastid changes reported by HRUBÝ (8) in 1935. The work of HRUBÝ is particularly pertinent to my work. HRUBÝ, while associated with the late Dr. BROŽEK of Prague, exposed spores of *Equisetum arvense* to filtered radiation. Using an aluminum filter 0.5 mm. in thickness, amoeboid plastids of the type that I have described occurred in a high percentage of prothallia. With an exposure of spores to 2355 r units, 21.6 per cent of the prothallia possessed amoeboid plastids, while an exposure to 6800 r units resulted in amoeboid types being found in 46.5 per cent of the prothallia. No other types of plastids were reported. It will be noted that the frequency of prothallia exhibiting plastid abnormality is extraordinarily greater than in my work. This may be due to the fact that the spores of *Polypodium* are low in water content, while the spores of *Equisetum* appear to be in a much more active state physiologically and to have a higher water content. HRUBÝ makes no statement concerning the permanency of these plastid changes. It is probable that continued growth of the prothallia was not possible under the cultural methods he employed.

That cytoplasmic alterations may occur as a result of X ray treatment is evident from the results of various investigations, which have been summarized by GOODSPEED and UBER (4). Of particular significance here is the work of BIEBLE (1), who exposed *Bryum capillare* to a radiation. Agglutination and destruction of plastids followed. NADSON and ROCHLIN (19) state that the chondriosomes (probably proplastids) are more sensitive to X rays than is the nucleus. In their work, the chondriosomes of the epidermis of the onion scale after exposure to X rays swelled, became modified in shape, and fragmented.

In his monograph on the pathology of chloroplasts, KÜSTER (15) summarizes the work on plastid abnormalities. For the most part abnormalities were induced by mechanical or chemical means. Centrifuging, treatment with solutions of various alcohols, chloralhydrate, etc., resulted in various plastid modifications; but concerning the persistence of these changes no data are given. One of the more interesting cases was reported by KÜSTER (14) for *Bryopsis plumosa*. Plants cultivated in the laboratory for many months showed striking abnormalities in their plastids. Some filaments had normal plastids, others had long bandlike plastids, the greatest length being  $280\ \mu$ ; other plastids were hook-shaped, amoeboid in form, or otherwise modified. In *Spirogyra* also plastid changes were noted.

These forms and those described by KÜSTER in his monograph—fusion, incomplete division, abnormal and unequal division, swelling, and so-called vacuolization—occur in the types that I have described. The significance of the work by KÜSTER in relation to my work is merely that the plastids are capable of markedly modifying their form under certain conditions. KÜSTER gives no evidence for the permanency of plastid changes reported by him.

Whether or not these changes are determined by chromosomal changes or are entirely independent of them cannot be stated at present. Cytological work on some of these forms is now in progress. That the plastids may differ independently of genic differences is apparent from the work of CORRENS and others. The following quotation from SINNOTT and DUNN (26) summarizes clearly the present status of the subject:

Particularly important among these are certain traits in plants involving chloroplast development and constituting the so-called "albomaculatus" types of leaf variegation, in which the normal green tissue is irregularly spotted with patches of paler green or white, a type of variation intensively studied by CORRENS and others. These may be small or may include entire leaves or branches. This character occurs in a wide variety of plants, and its inheritance has been determined in more than 20 genera. Flowers in wholly green branches produce seed which grow into normal plants; flowers on variegated branches yield offspring which have variegated foliage, and flowers from branches wholly white give progeny without chlorophyll; but in every case the source of the pollen has no influence on the offspring. Inheritance is wholly maternal. Variegation seems clearly to be determined by agencies localized in the cyto-

plasm rather than in the chromosome. A satisfactory explanation of the mechanism of inheritance for such a trait is available, however, since variegation is evidently the result of differences in chloroplast development and since the primordia of these bodies, from which the plastids of the whole plant are ultimately derived, are present in the cytoplasm of the egg.

It is emphasized by SINNOTT and DUNN that while about forty such cases are known, at least several hundred cases are known where the plastids and chlorophyll modifications are controlled by genes. SIRKS (27) also summarizes a few of the special cases in plastid inheritance.

IMAI (9), recognizing that the plastid character may be controlled by genes, also emphasizes that the plastid may mutate. This mutation may be determined by a gene within the plastid; this he terms the plastogene. "Through plastogene mutation variant plastids appear among otherwise homogeneous plastids of a cell, variegation being exhibited when they propagate into [in] the cells." He states also that this automutation of plastids in meristematic cells is hindered and is less common in well nourished cells. The mutation to which he refers involves chlorophyll content and not form of plastid. At gametogenesis reverse mutation occurs and the zygote has only green plastids. At present it would seem that IMAI's data are inadequate to support the views he expresses.

In the introductory paragraph the question was raised whether or not the plastid changes are independent of any chromosomal alteration or aberration. No direct answer to this question is as yet possible. In my work great variation of plastids within a cell of a single type may occur. This is suggested evidence against genic control. In the case of the grouped type the behavior of the plastids in the sporophyte and succeeding gametophyte seems opposed to genic control. The plastids in this type are grouped about the nucleus, and in all prothallia derived from the original prothallium with grouped plastids this condition prevails. Various sporophytes have been obtained as a result of sexual reproduction, from prothallia of the grouped plastid type. Sporophytes have been obtained from cultures 121, 96, 187, and 188, and all have possessed the same plastid characteristics. At the very outset the young leaves and the stem have chloroplastids which are grouped about the nucleus, just

as in the gametophytic stage. Even the plastids in the guard cells of the stomates are grouped about the nucleus. This grouping occurs in all leaves produced during the first 2-3 years. The first leaves are several mm. to 2 cm. in length; by the third year leaves are produced which may be 6 inches in height. When the adult form of leaf is produced, usually after the third year, the characteristic grouping no longer prevails. These leaves, and others produced subsequently, appear to have their plastids distributed in the surface cytoplasm just as in the normal type. The plastids still remained grouped in the leaves produced during the first 3 years. The same plant may therefore have what may be termed juvenile leaves with grouped plastids and adult leaves with plastids normally distributed. Forty such juvenile leaves may be present with grouped plastids. In the epidermal cells of these adult leaves occur leucoplasts which still remain grouped, but this is the only evidence of such condition.

No spores have been obtained from the juvenile leaves but only from the adult. Thus far spores have been obtained from cultures 121 and 188 of the grouped plastid type. Prothallia produced from these spores are entirely normal in appearance. The grouped characteristic which has persisted—and still persists in prothallia derived from the original prothallium with grouped plastids—disappears in the sporophyte when it is 2-3 years old and does not reappear in the new gametophyte.

Little can be said at present regarding the cause of this loss. The geneticist would perhaps be inclined to consider that a remutation had occurred, or that the expression of the gene or genes controlling plastid behavior is modified by the internal environment of the adult leaves.

The grouping of plastids appears to be similar to an agglutination phenomenon. It is conceivable that a substance is produced in the cell by X irradiation which is responsible for the grouping, and that as later stages of the sporophyte are reached this agglutinating substance is dissipated or no longer produced.

In the case of the giant plate type (no. 262) the sporophyte generation again exhibits the giant plate type of plastid. These remarkable plastids are therefore transmitted as a result of sexual reproduction to the sporophyte and to the succeeding gametophyte. This inheritance

would be in accordance with the usual genetic behavior. But even here the plastid type could be independent of the gene. In other cases there appears to be a partial reversion of abnormal types to normal, but with some individuals retaining the abnormalities. These also tend to confirm the view of independent behavior. These cases will be considered in a subsequent paper.

From a purely physiological point of view, plastid mutation should occur as a result of X irradiation. If it is assumed that there is continuity of plastids from one generation to another, such mutations should become permanent. How these mutations could occur is of course still in the realm of speculation. In the spore the plastids are present as plastid primordia in the cytoplasm. The exact number that may be present has not as yet been definitely determined. The number of plastids in the basal cell of the protonema is generally ten or less, and it is probable that this is the number of plastid primordia in the original spore. These plastid primordia are protoplasmic; and as indicated by the work of GRANNICK (7), chloroplasts are high in protein. It has been demonstrated by NORTHRUP (20) that crystalline pepsin is deactivated by  $\beta$  rays from radium and that other enzymes are deactivated by exposure to rays of high frequency. Flocculation is an obvious effect of high frequency (6). The significant point is that proteins are changed, and since proteins constitute a large proportion of the plastid body, changes in the protein would probably bring about changes in the plastid. Of course changes in the proteins of the plastid may be only incidental to other chemical changes occurring there. Equally pertinent to the mechanism of plastid mutation is the interesting concept of FRICKE that the points of primary activation by X rays and the points of chemical reaction are usually separated in space. If in the spore the chemical activation is in the cytoplasm, it is possible that all proplastids would be modified, and this could account also for the grouping of plastids previously described.

It seems a reasonable supposition that the extraordinary changes in plastids may be associated with changes in the protein of the plastid. With these protein modifications, changes in surface tension and viscosity would follow, with change in form and size resulting. A new condition once established continues by plastid divi-

sion. This presupposes also that the altered substances, be they proteins or other substances, are resynthesized in the pattern of the altered material. This is probably analogous to the multiplication of a virus once it is established in a suitable host.

Further studies of a physiological nature are to be undertaken with these plastid types. Their photosynthetic efficiency should provide information of interest. Studies on the inheritance of these plastid types is progressing and it is expected that results of these latter studies may be published soon.

### Summary

1. The spores of *Polypodium aureum* may be treated with calcium hypochlorite up to 50 minutes at least, with no deleterious effect on germination and growth.

2. Irradiation of spores with dosages as high as 30,000 r units is without effect on the percentage of germination. With higher dosages the percentage of spores germinating decreases. The growth rate is decreased with dosages as low as 10,000 r units, and progressively decreases with increased dosage.

3. From seventy-two cultures in which were sown nearly 300,000 spores, fifty-one prothallia having abnormal chloroplasts were isolated. Twenty-seven prothallia survived. These have been classified according to the characteristics of the chloroplasts. Including the normal, eleven types were noted. These prothallia have been propagated vegetatively during a period of 7 years. The plastid types persist.

4. Certain types are transmitted to the sporophyte as a result of sexual reproduction and retransmitted to the new generation of the gametophyte.

5. The grouping of the plastids (type B) disappears in the adult leaves of the sporophyte and does not reappear in the new gametophyte generation.

6. On theoretical grounds it is suggested that the plastid changes may be independent of any genic changes, and that certain plant mutations may be due solely to plastid changes.

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# SPORE GERMINATION AND VEGETATIVE STAGES OF THE GAMETOPHYTES OF HYMENOPHYLLUM AND TRICHOMANES

ALMA G. STOKEY

(WITH THIRTY-FIVE FIGURES)

## Introduction

In 1843 TASCHNER (23) gave a short account of the germination stages and young gametophytes of *Trichomanes schmidianum* and *T. capillatum*, based on material collected in the indusia of dried specimens. PRESL (20) in the same year published a few drawings of germinating spores but no later stages. Later METTENIUS (18), in connection with an account of the sporophyte, gave an extensive account of the gametophytes of *Hymenophyllum* and *Trichomanes*, based largely on dried herbarium material. He examined the germination stages of more species than has anyone since his time. He also studied *H. tunbridgensis* in culture. PRANTL (19) described and gave figures of early stages of *H. elegans*, *T. speciosum* (*T. radicans* Sw.), and *T. schmidianum*. JANCZEWSKI and ROSTAFINSKI (17) discussed the mature prothallia, sex organs, and embryo of *H. tunbridgensis*. GOEBEL (12) described early stages of the gametophyte obtained in cultures of *H. dilatatum*, *T. diffusum*, and *T. palmatifidum*, and of field collections in Java of older stages of *H. dilatatum*, *H. eximium*, and *H. smithii*. BOWER (1) described the mature gametophytes of *T. pyxidiferum* and *T. alatum* from material growing in the greenhouses at Kew and Edinburgh, and gave an account of aposporous and apogamous growth in *T. alatum*. Later he gave a brief account of apospory in *T. kaulfussii* Hk. and Grev. (2). GIESENHAGEN (11), in a discussion of the Hymenophyllaceae, described some gametophytes chiefly from collections made by GOEBEL. SADEBECK (21) discussed and illustrated the germination of *H. rarum* and an undetermined species of *Trichomanes*. GEORGEVITCH (9, 10) reported on *T. kaulfussii*, with special reference to apogamy, apospory, and gemmae formation. HOLLOWAY (16) described the experi-

mental culture of the gametophytes of *H. pulcherrimum* and *T. reniforme*, and included results of field observations on several species of *Hymenophyllum* and *Trichomanes*.

### Material and methods

This study is based on cultures of seven species of *Hymenophyllum* and four of *Trichomanes* collected at Tjibodas and Lebak Saät, Java, early in July, 1937, and on a culture of *H. blumeanum* started in Formosa in 1931. The spores were collected from fresh leaves on smooth white paper and were planted within an hour or two after being shed. The early stages were obtained from cultures on sterilized decayed wood, from which it is easy to remove the young gametophytes. Several of the cultures on wood flourished for several months but eventually succumbed to infection by fungi. In a few cases early stages were also obtained from spores which had fallen into the indusium and germinated there. The later stages were obtained from cultures on peat by methods described in a previous paper (22). It may reasonably be inferred that conditions were favorable, since cultures of several species have proceeded to the stage of producing sex organs.

For a period of over six weeks the cultures were kept on a table near the window in the Treub Laboratorium, Buitenzorg, where they received approximately one-half the diffused light available. A few rough tests were made to ascertain a favorable intensity of light by comparing the growth of four sets of cultures of *H. holochilum*, *H. javanicum*, and *T. auriculatum* at different distances from the window. The intensity of light was measured by a metrophot. Station A was close to the window, where there was the maximum diffused light available in the laboratory; B, a table with one-half the light at A; C, with one-tenth the light at A; and D, with one-twentieth the light at A. In a 24-day test there was no difference in type of growth and very little in rate. This is, however, a short period for prothalli which grow as slowly as those of the Hymenophyllaceae. The slight advantage, more marked in *T. auriculatum* than in the more slowly growing species of *Hymenophyllum*, was in favor of those at station B where they received one-half the diffused light available through the window, with those at C (receiving one-tenth the maximum light)

next in rate of growth. These results, although meager and lacking in precision, indicate that the gametophytes of *Hymenophyllum* and *Trichomanes* are affected less by differences in intensity of light than are those of most leptosporangiate ferns. Their behavior in crowded cultures also suggested this.

A few rough tests were made on the length of viability of the spores of *H. javanicum* and *T. maximum*. The spores were collected on glazed paper and the paper folders were kept in a tin box at room temperature at the Treub Laboratory. After thirteen days about half the spores of *H. javanicum* were still alive; a few germinated when nineteen days old, but none after that. Spores of *T. maximum* were not viable after six days. Undoubtedly the spores would retain their viability for a longer period with a more careful technique in handling them.

After six weeks' growth in Java the cultures were brought to the United States. Further growth was followed in South Hadley, Massachusetts, where the cultures grew in the north window of a laboratory during the college year. During the three summer months they were at the Marine Biological Laboratory, Woods Hole, Massachusetts.

### Investigation

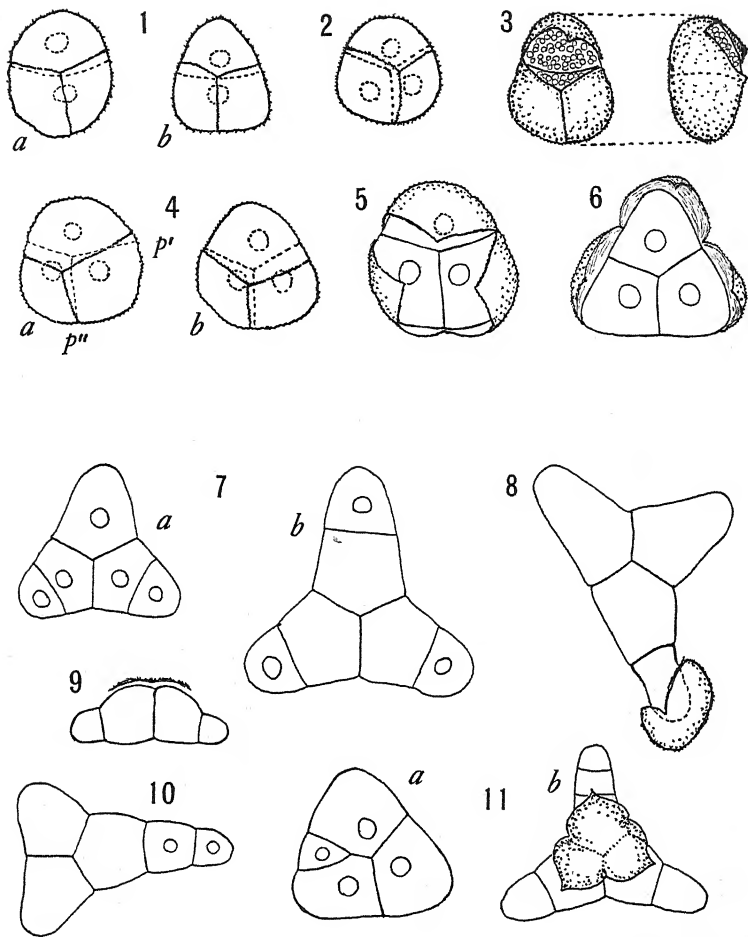
#### HYMENOPHYLLUM

Cultures of seven species of *Hymenophyllum* were started in Java. After two years the following are still flourishing: *H. acanthoides* Ros., *H. holochilum* (v.d.B.) C. Chr., *H. kurzii* Prantl, and a form which Dr. Posthumus said was probably *H. blumeanum* Spr. and which will be referred to by that name. *H. junghuhnii* v.d.B. was in culture only a few weeks; *H. fuscum* (Bl.) v.d.B. for eight months, and *H. javanicum* Spr. for eighteen months. A culture of *H. blumeanum* started in Formosa in 1931 is still flourishing after eight years, during which time transfer cultures have been made about once a year.

The spores of all these species are of uniform type, spherical or tetrahedral, with contents strikingly green owing to abundant chloroplasts, and with a thin papillate coat on which the ridges are conspicuous. In all seven species the spores germinated within the

sporangium with the division into two or three cells (figs. 1, 2). This occurs in all the species of *Hymenophyllum* investigated. In most cases when discharged from the sporangium the spore showed the characteristic triradiate division into three cells, but cases of two cells were not uncommon, and in some cases there were three nuclei but two were not separated by a wall. The first wall divides the spore into two approximately equal cells, one of which broadens more than the other (fig. 1) and is then divided by a wall which meets the first in the center of the spore (figs. 2, 4). The growth is such that the gametophyte takes on the form of a triangular cushion consisting of three cells separated by walls making approximately equal angles (fig. 4b). The position of the walls is such that they have been referred to as three walls which meet in the center, although METTENIUS gave a figure of the two-celled stage and pointed out that the walls do not arise simultaneously. It is often possible to distinguish the order of formation of the two walls (which will be referred to as primary walls), since the angle made by the bending of the first wall may remain for some time larger than the other two angles (fig. 4a) and the constriction on the outer wall may be more marked than that produced by the second wall. The triradiate walls lie under the ridges of the spore coat (figs. 2, 4); and after its rupture, which may occur within a few hours after planting, the three arms of the young prothallus grow in the direction of the longitudinal axes of the valves (figs. 5, 6, 11b). The three cells then enlarge noticeably, and much larger and sharper tips are formed (figs. 6, 8). Secondary divisions of the cells may not occur for several days, five to ten days in the cultures at Buitenzorg. This is slow in comparison with the rate of division in gametophytes arising from chlorophyll-bearing spores in other ferns, for example, *Osmunda* spp., *Todea barbara*, and *Onoclea sensibilis*, or even in comparison with the development of most leptosporangiate prothalli after germination.

The secondary walls, which cut off the three points or arms, follow in sequence (fig. 7), and the three may be complete within seven or eight days, or not until twelve to fifteen, or even longer (fig. 7). Prothalli with an abundant supply of chloroplasts and every visible indication of vigor may remain for a long period with surprisingly little activity in the production of new cells. In a rather crowded

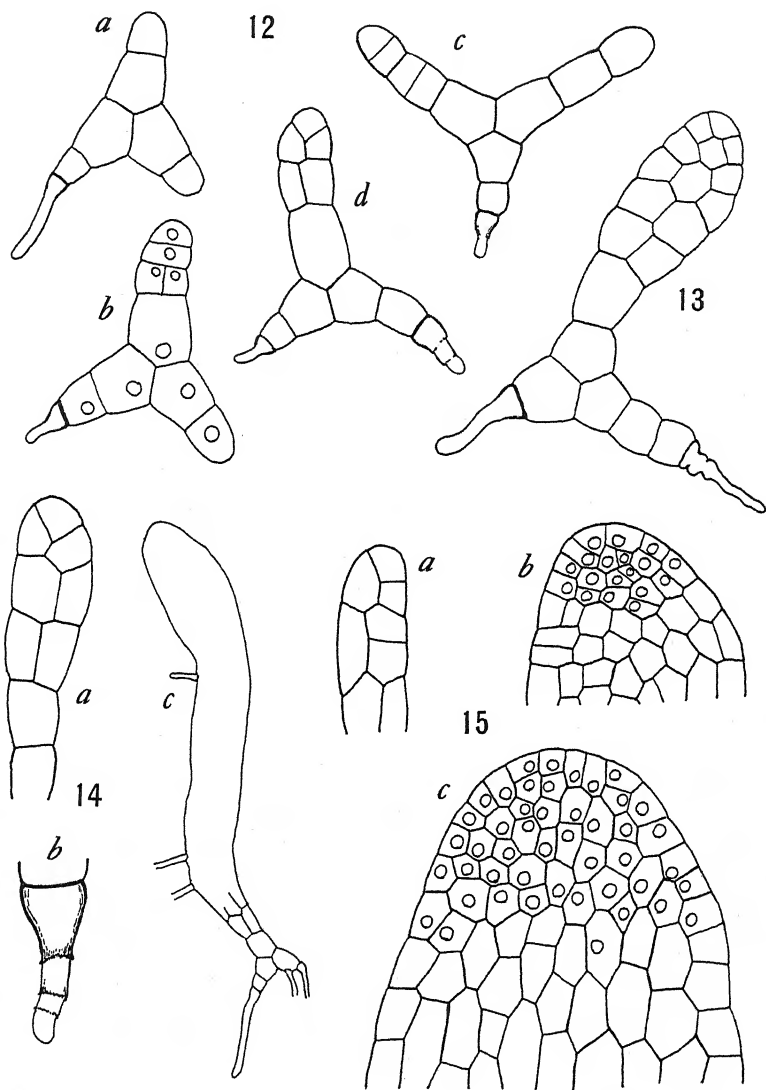


FIGS. 1-11.—Germination stages of *Hymenophyllum*. Fig. 1, *H. kurzii*, freshly discharged spores with first primary wall. Fig. 2, *H. acanthoides*, same with both primary walls. Fig. 3, *H. javanicum*, two views of germinating spore with first primary wall; 2 days old. Fig. 4, *H. acanthoides*, spores 3 hours after discharge from sporangium: *p'*, first primary wall; *p''*, secondary primary wall. Fig. 5, *H. javanicum* 1 day after planting. Fig. 6, *H. acanthoides* 7 days after planting. Fig. 7, same 8 days after planting. Fig. 8, *H. junghuhnii* 21 days after planting; position of spore coat unusual. Fig. 9, *H. holochilum*, side view of 6-celled prothallus. Fig. 10, *H. acanthoides* 21 days after planting. Fig. 11, *H. blumeianum*: *a*, unusual division in freshly shed spore; *b*, 21 days after planting; the three tips are in usual position with reference to valves of spore coat.

culture of *H. holochilum* nearly four months old, many prothalli were found which had only seven to ten cells and one or two rhizoids; but the cells had a full complement of chloroplasts and there was no suggestion of the depauperate aspect usually shown by retarded prothalli of other ferns grown in crowded cultures or in weak light. Healthy bright green prothalli of *H. acanthoides* consisting of only six cells were found in cultures five months old. The ability to remain vigorous in crowded cultures for so long is probably related to their adaptation to a wide range of light intensity. Irregularities, such as the production of a four-celled plate, were found occasionally (fig. 11a). Certain other irregularities occasioned by the failure of the second primary wall to develop will be discussed later.

Each of the three tips of the gametophyte grows independently into a rhizoid or a green filament. The branch most favorably placed continues as a filament for a short period and one or both of the others will be checked by the formation of a rhizoid which is usually terminal. When division in one arm is checked, the others may continue as in figure 12c, or even until there are longitudinal divisions in both branches; but ultimately one dominates and the other is checked. Although the early stages have a remarkably high degree of uniformity, subsequent development is varied, and, as noted by HOLLOWAY (16), "there is here no constant sequence in development such as is found in the fern gametophyte of the cordate type." Growth of the primary filament or filaments is usually apical but may be intercalary also in the early stages (fig. 12c). Longitudinal divisions appear first in the terminal cell, or less frequently in one farther back (fig. 12b). As all who have had *Hymenophyllum* gametophytes in culture have remarked, growth is very slow, not only in comparison with the quick germination but with the development of gametophytes of other leptosporangiate ferns. In the most rapidly growing culture, that of *H. acanthoides*, the largest prothallus noted had only twelve cells at the end of twenty-one days, and very few prothalli had more than twenty cells at the end of six weeks. In that time under similar conditions a polypod gametophyte might mature and produce a sporophyte.

Rhizoid development was late in all species. METTENIUS found rhizoid development belated in his cultures and in prothalli growing



FIGS. 12-15.—Fig. 12, *H. acanthoides*: *a*, *b*, 21 days old; *c*, 40 days old; *d*, 66 days old. Fig. 13, *H. holochilum*, 3 months old. Fig. 14, *H. acanthoides*: *a*, tip with apical cell, prothallus 3 months old; *b*, rhizoid from prothallus 26 days old; *c*, prothallus 5 months old. Fig. 15, *H. holochilum*: *a*, tip of branch 5 months old; *b*, tip with apical cell; *c*, tip with marginal meristem.

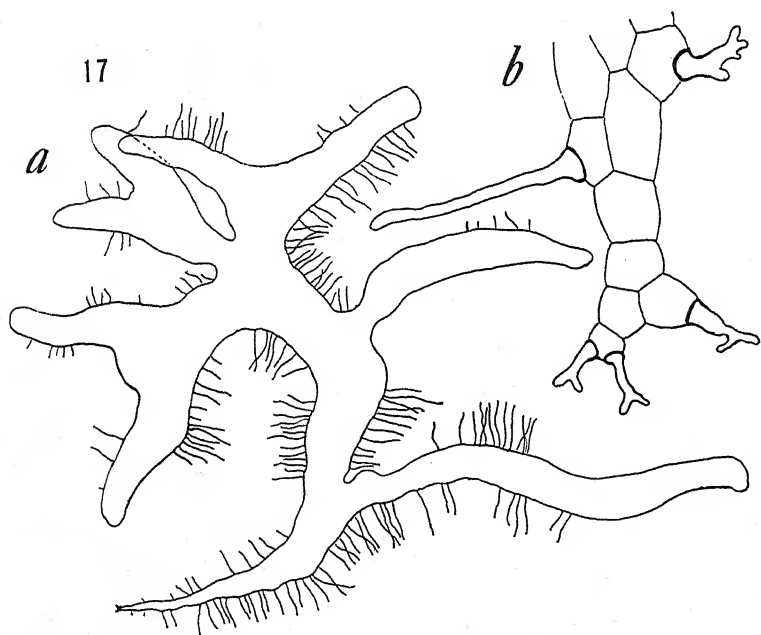
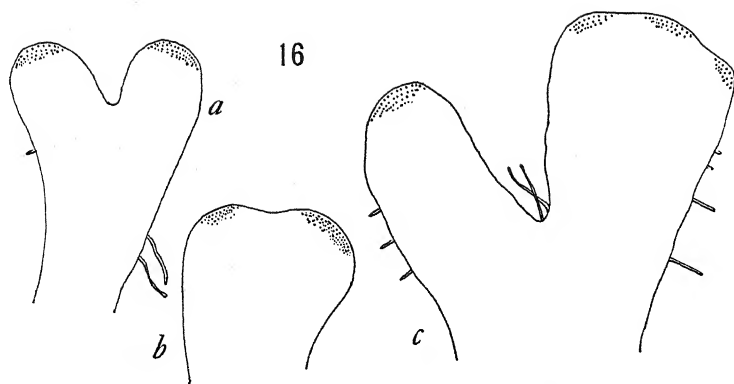
within the indusium, and gave figures of prothalli with seven to ten cells and no rhizoid. The earliest suggestion of a rhizoid was noted in *H. acanthoides* eight days after planting, but in most cases it was fifteen to twenty days. The first cell cut off from a tip may develop into a rhizoid (figs. 8, 13), but that is less common than the formation of a rhizoid from the second or even the third cell (figs. 12a-d, 13). The beginning of a rhizoid is indicated by the slender form of an elongating terminal cell or of a lateral papilla and by a change in the character of its contents. The young rhizoid on young plants is well provided with chloroplasts, which gradually grow smaller and paler, finally disappearing. The wall which separates the rhizoid from the green prothallial cell turns brown early, and the lateral walls begin to turn brown while there are still chloroplasts in the rhizoid. In a rhizoid which grows slowly there is often a colorless tip on a pale extension of the inner layer of the rhizoid wall (fig. 12c, d). In some cases there is a series of brown rims indicating where the inner layer has stretched beyond the inclosing layers (figs. 12d, 14b). Branching rhizoids are common, especially near the base of old prothalli. The branching rhizoid seems to be a general characteristic of the Hymenophyllaceae and is mentioned or illustrated in almost all accounts of the gametophyte, from TASCHNER's to the present. Two rhizoids may arise from one prothallial cell; such cases are frequent, particularly near the base of old prothalli (figs. 14c, 17b).

The broadening of one arm is usually brought about by the development of the terminal cell as an apical cell with two cutting faces (figs. 12d, 14a), but the behavior at the tip is not entirely like that of the apical cell of cordate prothalli, which gives rise to segments undergoing many divisions and considerable enlargement. At an early stage in the development of the ribbon-like prothallus the terminal cell may divide by a wall more or less oblique to the longitudinal axis; but the segments may not divide at all, or at most a few times, and in any case they enlarge very little (figs. 12d, 14a, 15b). The small number of cells formed by the segments and their limited growth prevent the attainment of a lobed tip. Sometimes the wall in the terminal cell is more nearly parallel to the axis and there is formed a tip in which is a series of vertical and cross walls (fig. 15a). In any case the apical cell, such as it is, is succeeded by a

group of meristematic marginal cells (fig. 15c). The tip remains rounded or blunt, even when the ribbon-like branch becomes twenty to thirty cells wide (fig. 16).

Branching is ordinarily brought about by cessation of the activity of some of the cells of the rounded meristematic apex with continued activity of the cells on both sides of the subsiding meristem (fig. 16b). Occasionally the checking of the activity of the central cells is followed by development of rhizoids in the stretch of margin between the new branches. If the cells which cease division are median, the two meristematic regions approximately equal, and the new branches grow at the same rate, the result is a dichotomy (fig. 16a). Or two regions at the tip may cease activity, leaving three meristematic areas which will result in three branches. Or, as is usually the case, the meristematic regions may be unequal; then the two branches may both elongate at much the same rate, making a strong and a weak branch, or one branch may lag and be left behind by the greater growth of the other, becoming a lateral branch (fig. 16c). As the branches lengthen they twist in such a way that they do not all lie in one plane, but form a rosette-like tuft. A characteristic gametophyte one year old of *H. holochilum* is shown in figure 17a. The original three primary cells were still present (fig. 17b), and the activity of horizontal branches which give rise to a succession of vertical branches had not yet begun. In these cultures the vertical branches were usually 9–12 mm. long and fifteen to thirty cells wide. The primary cells die after the prostrate branches become active, and none were found on prothalli two years old. Adventitious shoots may arise at any place on the margin of the mature portions of the thallus, but are found most frequently on the lower parts, particularly on the prostrate branches. They are produced freely and arise in the same manner as such branches on other ferns.

Rhizoids are abundant on the erect branches, even 10–12 mm. from the base (fig. 17a). They arise from the marginal cells only and usually grow toward the under side. All the rhizoids except those on the basal and lower parts of the branches stand far above the substratum and are far too short to reach it, as HOLLOWAY noted for *H. pulcherrimum* in culture and for that species and others in the field. It is not apparent that they are of any use to the gametophyte—un-



FIGS. 16, 17.—Fig. 16, *H. acanthoides*, tips of branches with position of marginal meristem indicated by stippling. Fig. 17, *H. holochilum*: *a*, prothallus 1 year old: *b*, base of *a*.

less they are effective in keeping space between the overlapping lobes and branches in crowded tufts. The presence of an endophytic fungus has been noted by various writers, but none was found in the cultures which were planted on sterile peat. The vigorous growth of the gametophytes with uninfected rhizoids gives support to the statement of HOLLOWAY (16) that "the fungal infection is only of minor importance in these gametophytes."

The branching ribbon-like gametophyte is the only type ordinarily associated with *Hymenophyllum*, although METTENIUS described that of *H. microcarpum* as consisting of filaments with profuse branching which suggested dichotomy. He described the gametophyte of *H. pulchellum* also as being prevailingly filamentous, with leaflike expansions two to five cells wide, an appearance similar to that of *T. sinuosum*. GOEBEL (12) raised the question of the identity of these gametophytes, since they differed so much from those which he himself had collected and from the others described by METTENIUS. As a third type, METTENIUS described the branching ribbon-like gametophyte of *H. elegans*. This is apparently the prevailing form in *Hymenophyllum*.

The gametophytes of *H. javanicum* (fig. 18) and those of *H. fuscum* suggest the aberrant filamentous type which METTENIUS described. The germination of these two species was like that of all other recorded species of *Hymenophyllum*, but growth was slower and development was of a different type from that described for *H. acanthoides*, *H. holochilum*, *H. kurzii*, and *H. blumeianum*. The filamentous development continued for many months, with abundant branching, so that each gametophyte made a compact rosette. The cells in both species were short and swollen, and there were many short stubby branches, but they did not suggest a dichotomous type. There was a strong tendency for the primary cells to make intercalary divisions and to produce extra rhizoids and filaments, although in other species of *Hymenophyllum* such activities are rare.

*H. fuscum* was in culture eight months and in that time no expansions were formed, although there were longitudinal divisions in a few cells. In cultures of *H. javanicum* sixteen months old most of the branches were of the filamentous type, but branches two cells wide

were found on a few prothalli although there was no indication of the formation of an active apical region (fig. 18c).

It is difficult to keep such forms in culture as they are more likely to be overcome by chance infections of algae or fungi than are those which grow more rapidly. It is possible that this unusual type of development should be attributed to unfavorable conditions, but it

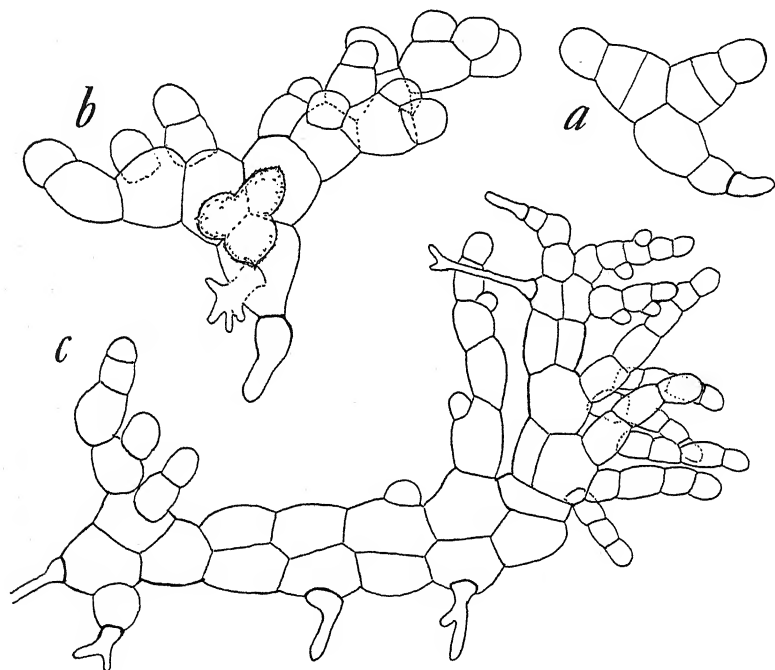


FIG. 18.—*H. javanicum*, stages in development of prothallus: a, 22 days old; b, 8 months old; c, 16 months old.

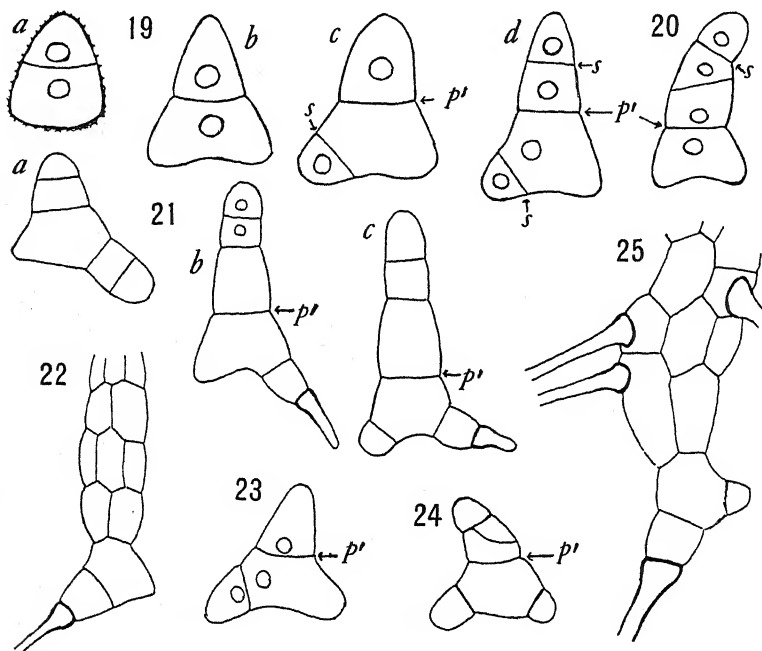
does not seem probable that such is the explanation. Individuals of other species which grew slowly did not follow this line of development. HOLLOWAY (16) found that the gametophyte of *H. pulcherrium*, which ordinarily produced branching ribbons, tended to be filamentous when grown in specially moist conditions. The gametophytes of *H. fuscum* and *H. javanicum* were grown under the same conditions as those of the other species, and there was considerable variation in the amount of moisture in the peat at different times, although at no time were they very moist.

The gametophytes of *H. holochilum* and *H. kurzii* began to bear sex organs when about twenty months old. A few scattered antheridia were found near the margin, with archegonia singly or in twos on the same gametophyte. COPELAND (8) includes *H. kurzii* in *H. holochilum*. Although the cultures of *H. acanthoides* grew more rapidly than any of the other species at the beginning, neither that nor the Java cultures of *H. blumeinum*, both two years old, have as yet produced sex organs. A culture of *H. blumeinum* which was started in Formosa in August, 1931, is still flourishing. When nearly four years old it began to bear archegonia and has continued a sparing production of them at intervals ever since. No antheridia have been found in the cultures.

An interesting variation in germination was found in *H. acanthoides*, *H. holochilum*, *H. kurzii*, *H. fuscum*, and *H. javanicum*. It occasionally happens that the second of the primary walls fails to develop and the typical radiate three-celled stage is not attained. Figure 19a shows a form which is occasionally found on the rupture of the sporangium, and at this stage it cannot be determined whether the second wall is belated or suppressed. Figure 19b, however, shows a prothallus of *H. holochilum* sixteen days old in which the second wall has probably been suppressed, as ordinarily the division into three cells takes place shortly after the spores are discharged, if (as is usual) it has not already taken place in the sporangium. When the secondary primary wall does not appear, the first will remain flat and does not assume its characteristic angle; but for a short period at least it lies not far from the center of the original cell. When the second of the primary walls is suppressed, growth of the three points may proceed in the usual way, but in general there is less uniformity of development. The two tips belonging to the undivided cell enlarge less than the other, and frequently one of these remains inactive and does not cut off a peripheral cell.

The primary wall (*p'*) is considerably broader than the secondary wall (*s*), often twice as broad. The primary cell cut off by the primary wall may undergo secondary divisions before the peripheral divisions take place in either of the other two arms (fig. 20), as sometimes happens in prothalli with typical germination (fig. 10). In the gametophytes represented in figures 21a, b; 22, and 23 the secondary walls in the third tip are overdue, according to the usual rate of de-

velopment, and they are probably cases in which not only is one primary wall suppressed, but also the secondary wall in one tip. In the usual type of germination it is rare to find a gametophyte which



FIGS. 19-25.—Prothalli with first primary wall present but second suppressed:  $p'$ , first primary wall;  $s$ , secondary wall. Fig. 19, *H. holochilum*:  $a$ , freshly discharged spore with first primary wall;  $b$ , prothallus 16 days old;  $c$ , 16 days old, secondary wall in one tip;  $d$ , 16 days old with two secondary walls. Fig. 20, *H. acanthoides*, 21 days old, primary and secondary walls in one tip; intercalary division; two tips without secondary walls. Fig. 21, same:  $a$ , 3 months old, secondary walls in two tips;  $b$ , 26 days old, secondary walls in two tips (one undivided);  $c$ , 57 days old, secondary walls in all tips. Fig. 22, *H. kurzii*, base of prothallus 5 months old, one tip without secondary wall. Fig. 23, *H. fuscum*, 12 days old. Fig. 24, *H. javanicum*, 51 days old, secondary walls in all tips; intercalary wall in primary cell. Fig. 25, *H. holochilum*, base of prothallus 1 year old; late stage of prothallus like fig. 21c.

has reached the plate stage without at least one division in the other tips of the original three-celled plate, but it is much less rare in the type under discussion. In figure 22 is shown a gametophyte which is evidently a later stage of one which began like the one in figure

21b; figure 25 shows one which probably began like that in figure 21c. The relation of this type of germination to that of *Trichomanes* will be discussed later.

#### TRICHOMANES

Four species of *Trichomanes* were studied. *T. auriculatum* and *T. bilabiatum* have been in culture for two years and have reached the stage of bearing sex organs. *T. maximum* has also been in culture for two years but it has grown much more slowly and has borne

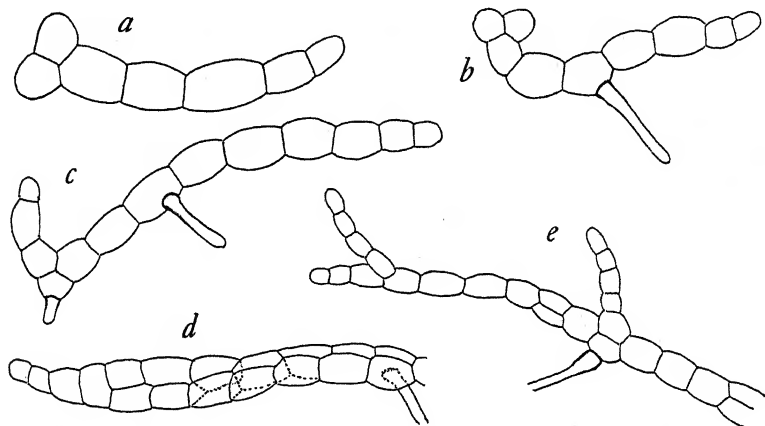


FIG. 26.—*Trichomanes nitidulum*: a, b, c, prothalli 4 months old; d, 8 months old; e, branch from prothallus 1 year old.

antheridia but not archegonia. *T. nitidulum* was in culture for twenty months.

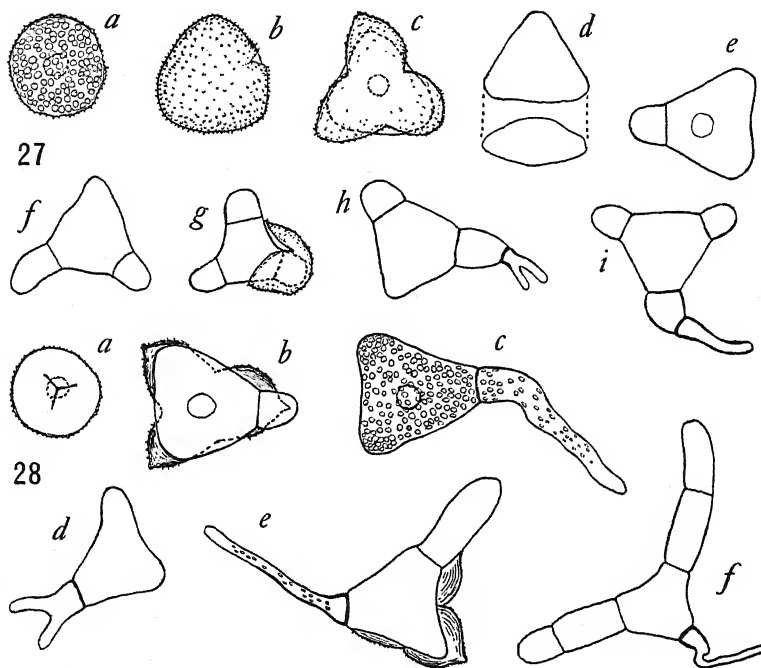
*T. nitidulum* in its germination follows the type of *Hymenophyllum* in the formation of three cells separated by radiating walls (fig. 26a-c). This type was recorded by METTENIUS for *T. ampliatum*, *T. crispum*, and *T. reniforme*. GIESENHAGEN (11, p. 422) was in error in stating that METTENIUS thought that only one type of germination was found in *Trichomanes*, since METTENIUS (18, p. 491) expressly states that the three species mentioned agree with *Hymenophyllum* in mode of germination and are unlike *T. schmidianum*, *T. olivaceum*, and *T. humile*, in which a small cell is cut off from each tip by a tangential wall. GOEBEL (12) recorded the 3-celled type for *T. palmatifidum*, and CAMPBELL (5) for *T. draytonianum*. The three

primary cells in *T. nitidulum* may give rise either to filaments or to rhizoids, as in *Hymenophyllum*, but in these cultures there was frequently considerable delay in the development of one or two of the tips (fig. 26a, b). In its subsequent growth the prothallus does not follow the usual *Hymenophyllum* development, but forms branching filaments. In cultures eight months old longitudinal divisions were found in single cells and in sections of filaments (fig. 26d), but no true ribbon-like expansions with apical broadening at the tip were found in cultures twelve to twenty months old. The prothallus showed considerable similarity in habit to those of *H. fuscum* and *H. javanicum*, although there was a difference in aspect because of the greater elongation of the cells and filaments in *T. nitidulum*.

The spores of *T. auriculatum*, *T. bilabiatum*, and *T. maximum* germinate in the manner usually associated with *Trichomanes*, or a modification of it. The spherical spores are deep green with abundant chloroplasts, and the tripartite ridges on the thin papillate spore coat are short and inconspicuous (fig. 28a). Germination in the sporangium was not found in these species. TASCHNER, however, found in *T. schmidianum*—which in other respects is similar to these three species—that the spores may germinate within the sporangium, and he gave a figure of the two-celled stage. Cracking of the spore coat usually occurs within a day or two after the planting of the freshly discharged spores.

In *T. auriculatum* and *T. bilabiatum* a triangular cushion-like body develops after the rupture of the spore coat and before there are any septations (fig. 27b-d). As in *Hymenophyllum*, each tip of the spore ordinarily is in line with a valve of the spore coat (fig. 27c), and the growth from each tip follows the longitudinal axis of the valve (fig. 28b, e). If, as occasionally happens, the crack is shallow or the spore is crowded as it germinates, the spore coat may be found as a cap on one tip of the thallus (fig. 27g). Within two to four days the first wall may appear and cut off one of the tips (figs. 27e, 28b); a second tip is then cut off (fig. 27f), and ultimately a third (figs. 27g, i; 28f), leaving an undivided central cell. This will be referred to as the *Trichomanes* type of germination, since so far as is known it is found in no other genus. In the case of spores germinating and continuing growth within the indusium, on which much of the early work was

based, the three peripheral cells are more likely to be of the same size for a period after the tangential walls appear than in the case of spores germinating under conditions more favorable for growth. Stages such as that in figure 27g were found within the indusium or in cultures with slow growth, twenty-five to thirty-five days after

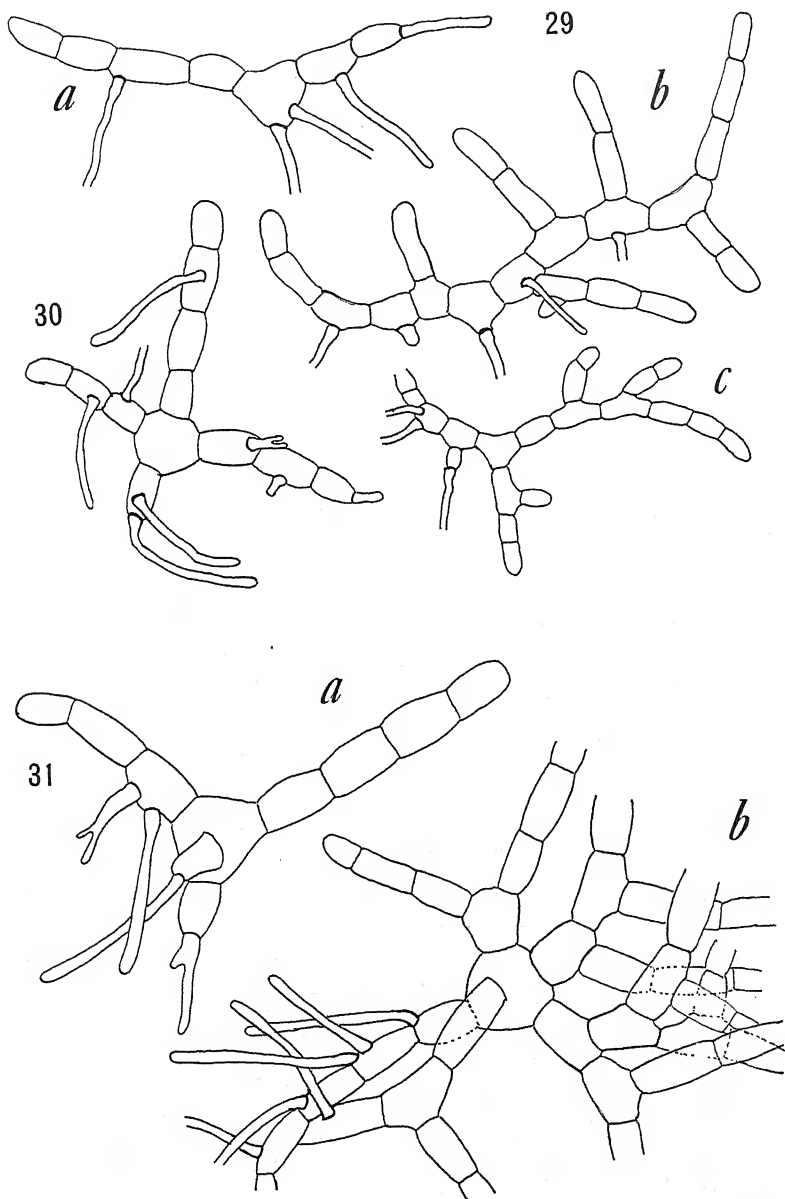


FIGS. 27, 28.—Fig. 27, *T. bilabiatum*: a, fresh spore; b–d, stages in germination before appearance of walls; d, 4 days old, two views; e, 4 days old; f, 5 days old; g, prothallus germinated within indusium, position of spore coat unusual; h, 34 days old; i, 43 days old. Fig. 28, *T. auriculatum*: a, fresh spore,  $\times 300$ ; b, 2 days old; c, 5 days old; d, 17 days old; e, 9 days old; f, 24 days old.

planting. In cultures on wood, where conditions were favorable, the second wall usually appeared when the prothallus was five to ten days old (fig. 27f), and the third at fifteen to twenty days, when the first arm and even the second had made noticeable growth. The growth of these cultures indicates that the more favorable the conditions for growth, the more variable and the less symmetrical the young gametophyte. Germination in *T. bilabiatum* was in general

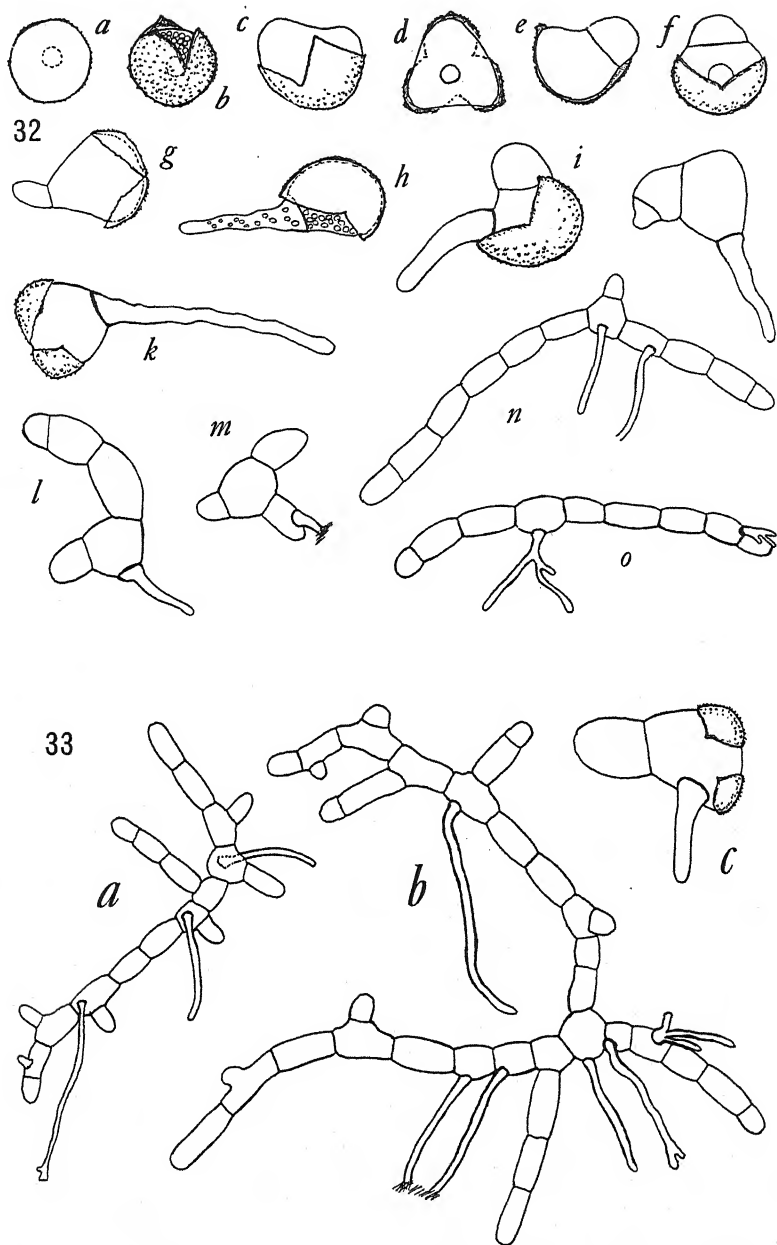
more symmetrical than in *T. auriculatum*. This may be due solely to its slower growth, or it may indicate that it is nearer to the central type of the genus. In *T. bilabiatum* the first rhizoid usually arises from the second cell formed from a tip, but in *T. auriculatum* the first cell cut off usually grows into a rhizoid (fig. 28c-e). Rapid growth of the first rhizoid usually prevents the attainment of the symmetrical 4-celled stage, although by the formation of walls in the three tips an undivided central cell is formed (fig. 28f). In *T. bilabiatum* the wall cutting off the third tip is sometimes belated, but no old prothallus was found in which the wall was suppressed. In *T. auriculatum* it is even more likely to be belated, but the third tip usually develops with considerable vigor in the course of time. The central cell remains green and retains its power to divide for many months. At a later period it usually gives rise to one or more filaments or rhizoids in addition to the three growths arising from the tips of the original triangular cushion. Gametophytes three to five months old usually had one or more rhizoids or green filaments arising from the central cell, in addition to the three growths derived from the tips of the spore. This tendency to form extra filaments and rhizoids was more marked in *T. bilabiatum* than in *T. auriculatum*, and gametophytes of the former species four to five months old usually had four or five filaments arising from the central cell (figs. 30, 31).

The mature gametophytes of both *T. auriculatum* and *T. bilabiatum* are of the branching filamentous type with no longitudinal divisions in the vegetative cells. The cells of the former species were usually longer and more slender than those of the latter, giving the plants a slightly different aspect under the microscope, although the general appearance of both cultures was the same. Both were equally green and equally vigorous, and to the unaided eye looked like a fine and healthy green turf, the *Rasen* of METTENIUS and GOEBEL. Antheridia appeared on the gametophytes of both species about nine months after planting, and a few weeks later archegonia had developed. Sex organs were produced more abundantly on *T. bilabiatum* than on *T. auriculatum*, and during the summer months numerous archegoniophores were formed on pale prostrate branches toward the periphery of the tufts. Antheridia were produced sparingly and no swimming sperms were seen; no sporophytes had developed at the end of two years.



FIGS. 29-31.—Fig. 29, *T. auriculatum*: a, prothallus 51 days old; b, 3½ months old; c, 4 months old. Fig. 30, *T. bilabiatum*, 4½ months old. Fig. 31, *T. bilabiatum*: a, prothallus 3 months old; b, central portion of prothallus 5 months old; primary cell with five branches.

The spores of *T. maximum* are smaller than those of the preceding two species but are similar in aspect. Although cracking of the spore coat occurred within a day or two after planting, the first wall was not found in any gametophyte until ten days later (fig. 32*e-h*). Triradiate development was found (fig. 32*d, g, k*) but it was hardly as common as the less symmetrical development (fig. 32*e, f, h*). The first cell cut off develops as a rhizoid in many cases, and there is a definite tendency toward suppression of the third tip, so that the gametophyte frequently develops as a more or less lopsided filament (fig. 32*o*). The prothallus of *T. auriculatum* sometimes develops for a considerable period in this manner but the modification of the triradiate type is less pronounced. It has been suggested (14) that the type of germination found in *T. maximum* should perhaps be regarded as a third type in the family, because previous descriptions indicate that only one cell is cut off at germination and then a straight filament develops. While there is a straight filament in many cases, however, there are also examples of triradiate development (fig. 32 *l, m, n*). The cutting off of a single cell at germination, with considerable delay in the formation of walls in the other two tips, is probably much more common in *Trichomanes* than has been supposed. Germination in *T. maximum* and related species probably represents an extreme case. While the extreme forms of the early germination stages of *T. maximum* are unlike those of such species as *T. schmidianum*, *T. diffusum*, and *T. bilabiatum*, there are many gradations found in *T. maximum*—from forms with extreme modifications to such variations from the triradiate symmetry as are frequently found in *T. auriculatum* and brought about by delay in wall formation in one tip of the thallus. In *T. maximum* itself triradiate development is eventually attained in many cases (figs. 32 *l, m, n*; 33*a, b*). It is possible that in some cases, perhaps in many, the first rhizoid does not arise as a growth from one of the three tips of the spore, as in figure 32*j, k*, but as an extra growth from the central cell such as is frequently found in older prothalli of *T. auriculatum* (fig. 29*a*). This is apparently the case in a prothallus which at sixty-eight days consists of only two green cells and a rhizoid (fig. 33*c*). Triradiate development with an extra rhizoid arising from the central cell is of common occurrence in prothalli five to seven months old. Unfortu-



FIGS. 32, 33.—Fig. 32, *T. maximum*: *a*, fresh spore; *b*, 2 days after planting; *c*, *d*, *e*, 10 days old; *f*, 15 days old; *g*, 11 days old; *h*, 20 days old; *i*, 34 days old; *j*, *k*, 26 days old; *l-o*, 5½ months old. Fig. 33, *T. maximum*: *a*, *b*, prothalli 7 months old; in each the primary cell has given rise to three filaments and a rhizoid; *c*, 68 days old.

nately the series made was not close enough to determine how early an extra rhizoid may arise from the central cell. The growth of *T. maximum* was extremely slow, as was noted by GOEBEL (13), who kept it in culture three years without obtaining sex organs. The largest prothallus found in December, 1937, in a culture five months old, had only twelve green cells and two rhizoids (fig. 32*n*). The rate of growth then increased, and in February prothalli were found which had twenty-five to thirty-five green cells and two to five rhizoids (fig. 33*a, b*). The drawings given by PRANTL for the closely related species *T. speciosum* (*T. radicans* Sw.) are very similar to some of the early stages of *T. maximum*. The similarity extends also to the rate of growth, which he described as *ausserordentlich langsam*. In cultures five months old he found no prothallus which had more than eight cells and two rhizoids. The older gametophytes developed as branching filaments with no longitudinal divisions, and in general aspect were like the preceding two species. Antheridia were found on gametophytes one year old but no archegonia have appeared on cultures two years old.

The rhizoids of all four species of *Trichomanes* are similar to those of *Hymenophyllum* in their heavy brown walls and their frequent branching. On young prothalli chloroplasts are abundant in the young rhizoids and may persist until the rhizoid has attained considerable length (fig. 28*c, e*). The production of two rhizoids by one cell is of common occurrence (figs. 29, 30, 31), and it is especially characteristic of the cells of the pale prostrate branches associated with the production of archegoniophores.

The gemmae, which develop abundantly and form a notable feature of the gametophytes of *Hymenophyllum* and *Trichomanes* under certain undetermined conditions, were not found in any culture.

### Discussion

All who have worked with the Hymenophyllaceae, either with the sporophyte or with the gametophyte, agree that it is a natural family rather sharply set apart from other leptosporangiate ferns. Among recent workers, BOWER (3) recognizes two genera, *Hymenophyllum* and *Trichomanes*. COPELAND (7, 8), dealing with the Old World genera only, recognizes three, returning *T. reniforme* to PRESL's genus *Cardiomanes*; he states that further divisions of the

family are probably desirable but should be deferred for the present. CHRISTENSEN (6) also recognizes *Cardiomanes*, and adds the South American monotypic genus *Serpyllopsis*. It may be well to consider to what extent the gametophyte may contribute to an understanding of the family. Considering the size of the family our knowledge is very limited. There are few species for which we have both germination and mature stages. The present discussion will consider only the vegetative character of the gametophyte, which according to our present knowledge shows a greater departure than the reproductive from other fern types.

Germination stages are known for about thirty species of *Hymenophyllum* (12, 16, 18, 19, 21) and for scarcely half as many of *Trichomanes* (12, 16, 18, 19, 23). They are remarkably constant in *Hymenophyllum* but decidedly less so in *Trichomanes*. A striking peculiarity of the gametophyte is its tendency to assume the triangular form, which is seen in *Hymenophyllum* before the secondary walls appear, and may be very striking in *Trichomanes* before any walls appear. There is no ready explanation for the triradiate habit. The filamentous habit in the family may perhaps be correlated with the hygrophilous habit; but it is difficult to see why the hygrophilous habit in this family should result in such beautiful radiate symmetry of form when in other families the development of filaments is associated with irregularities of growth and a high degree of plasticity, such as is found in the higher families of ferns. It is indeed true that in the filamentous species of *Trichomanes* there is greater irregularity and a higher degree of plasticity; but probably the filamentous habit in that case has been derived from a type with the conservative and symmetrical structure of *Hymenophyllum*. The triradiate tendency is one of the things which, in the present state of our knowledge of formative influences, must be looked upon as "inherent."

Among other peculiar features of the gametophyte of the Hymenophyllaceae are the pittings in the lateral walls of the cells of the gametophyte, described by various investigators (11, 12, 17, 21); the highly specialized gemmae which apparently develop abundantly in nature (2, 10, 14, 18); the rhizoids with their strong tendency to branch, and the restriction in position to the marginal cells of the ribbon-like prothallus.

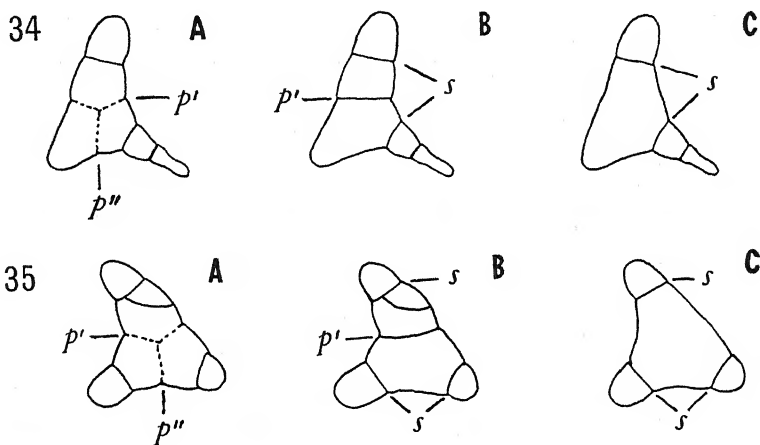
A general change in attitude toward the phylogenetic position of

the Hymenophyllaceae, and also of the relation of *Hymenophyllum* to *Trichomanes*, has occurred since PRANTL (19) characterized the family as *die niedrigste Entwicklungsreihe der Farne*. BOWER (3) has advanced reasons based on the morphology of the sporophyte for looking upon *Trichomanes* as being farther away than *Hymenophyllum* from the ancestral type. HOLLOWAY (16) arrived at the same conclusion from his study of the ecological habit and distribution of the New Zealand species. The greater plasticity and variability of the gametophytes of *Trichomanes* and the shorter time required for them to reach maturity suggest that *Trichomanes* is the younger and more specialized genus.

The relation of the two types of germination has long been a problem which has either been ignored or met by the statement that the walls which meet in the center of the germinating spore of *Hymenophyllum* no longer meet in *Trichomanes*, or vice versa (14, 19, 21). Such statements imply that the three walls which form in the tips of the triangular prothallus of *T. bilabiatum*—or a similar species—are homologous with the primary walls in *Hymenophyllum*. A serious objection to this interpretation is the fact that the primary walls in *Hymenophyllum* arise as two walls and not as three. It must be remembered that since the primary walls in the *Hymenophyllum* gametophyte arise under the tripartite ridges, the position of the primary walls is determined in the spore mother cell. The position of the three walls in the young thallus would not favor growth into the gaps made by the cracking of the spore coat, and therefore growth of the three tips proceeds along the longitudinal axes of the valves rather than into the gaps (figs. 5, 6). In *Trichomanes* there would be no structural difficulty in growth into the gaps, but even here the tips follow the longitudinal axes of the valves (figs. 27c; 28b, e; 32d). This suggests evolution from a form in which the direction of growth was determined by a condition which no longer exists. It is a strong reason for looking upon the 4-celled type of germination of *Trichomanes* as being derived from the 3-celled type of *Hymenophyllum*.

Certain types of germination which were found occasionally in several species of *Hymenophyllum* suggest a line of evolution from the 3-celled type of *Hymenophyllum* (and certain species of *Trichomanes*) to the 4-celled type of *Trichomanes*. This could be brought

about by the permanent suppression of the two primary walls which cause the triradiate division of the 3-celled spore. The two series of diagrams given in figures 34 and 35 suggest a probable sequence in evolution. The sequence in figure 34A-C is based on a camera lucida drawing (B) of a prothallus of *H. acanthoides* in which the first primary wall ( $p'$ ) is present but not the second; secondary walls ( $s$ ) have formed in the first primary cell and in one tip of the undivided



FIGS. 34, 35.—Diagrams suggesting evolutionary sequence of germination types in Hymenophyllaceae:  $p'$ , first primary wall;  $p''$ , second primary wall;  $s$ , secondary wall. Fig. 34B, *H. acanthoides*, 40 days old: A, diagram based on B, with usual arrangement of primary walls indicated by dotted lines; C, diagram based on B, with primary wall omitted. Fig. 35B, *H. javanicum*, 51 days old,  $\times 150$ : A, diagram based on B, with usual arrangement of primary walls indicated by dotted lines; C, diagram based on B, with primary wall omitted; intercalary wall also omitted.

portion. The same prothallus is shown in A, with the primary walls as they would ordinarily develop indicated by dotted lines. Diagram C shows the type of prothallus which would be found if both primary walls were suppressed. It will be seen that A is similar to the drawing of the same species in figure 12a, and C to the prothallus of *T. bilabiatum* shown in figure 27h. A similar sequence is shown in figure 35, based on a camera lucida drawing (B) of a prothallus of *H. javanicum*, in which the first primary wall ( $p'$ ) is present and there are secondary walls in all three tips. In A, as in figure 34A, the two pri-

mary walls ordinarily present are indicated by dotted lines; in *C* only the secondary walls are shown and the primary ones are omitted. (In *C* the intercalary wall present in the primary cell is not shown, as such walls are apparently limited to a few species of *Hymenophyllum* and are not found at all in *Trichomanes*.) Diagram *A* is not unlike figure 18*a* of the same species, while *C* suggests the prothallus of *T. bilabiatum* (fig. 27*g*).

There is no obvious ancestor for the type of gametophyte found in the Hymenophyllaceae. A strong similarity exists between the spores of this family and those of the Osmundaceae in form, chlorophyll content, and general aspect of the spore coat. There is also a degree of similarity in the early development of the plate. CAMPBELL (4) has figured a gametophyte of *Osmunda cinnamomea* with a triangular thallus not unlike an early 3-celled stage of *Hymenophyllum*, except for the rhizoid, and also figured *O. claytoniana* consisting of four cells without a rhizoid. The early appearance of the rhizoid is an indication of the polarity characteristic of most fern gametophytes but lacking in the Hymenophyllaceae. The writer has found young gametophytes with belated rhizoid development in both the species just mentioned, and also in *O. javanica* and *Todea barbara*. In cultures of *Marattia sambucina*, a fern of the deep rain forests of Java, the writer has occasionally found prothalli with five or six cells and no rhizoid. Delay in the development of the first rhizoid, rare in the more advanced families of ferns, is apparently not unusual in the Marattiaceae and the Osmundaceae. In subsequent development, however, the gametophytes of the Hymenophyllaceae have very little in common with those of the two families mentioned. Whatever the ancestors of the Hymenophyllaceae may be, we must assume that in the evolution of the type found ordinarily in *Hymenophyllum* there has been a great reduction in the thickness of the thallus, an elimination of the midrib, and an intensification of a slight tendency to lobe or branch found in many fern gametophytes. These changes may be related to growth in weak light and a moist environment, where filmy ferns are ordinarily found. Delay in the appearance of the rhizoid may be correlated with the hygrophilous habit of the family, although in the filamentous species of *Trichomanes* (*T. auriculatum*) the rhizoid may develop earlier than in *Hymenophyl-*

*lum*. This may be related to the shortening of the length of life of the gametophyte, which is indicated by the earlier maturity of *T. auriculatum*.

The relative uniformity of the early stages of the gametophyte of *Hymenophyllum* is followed by a period of greater variability and plasticity, but the mature stages again seem to be fairly constant in type. The ribbon-like expansions show variations in details of branching, but in general habit conform to a well defined pattern. The filamentous type with its modifications is, according to our present knowledge, not common in the genus. We have no information about its behavior at maturity. It is not known whether in any species of *Hymenophyllum* the filament comes to maturity and produces sex organs, as in the filamentous species of *Trichomanes*, or whether—as in most families of ferns—it is limited to the vegetative, or at most the antheridial, stage. At present it is known only in early developmental stages of *H. javanicum* and *H. fuscum*, and from the account by METTENIUS of older stages of *H. microcarpum* which had not yet produced sex organs. Apparently there has been a reduction from the plate to the filament in *Hymenophyllum* as well as in *Trichomanes*. This has probably occurred several times in the family, and, so far as now known, there is no reason for assuming that any close relationship links the forms in which it occurs. There is apparently considerable variation in the length of time required by different species to attain maturity. We do not know how constant this is, or whether it can be correlated with the phylogenetic group, or with the ecological habit.

The developmental history of the gametophyte is known for only a few species of *Trichomanes*. As there is apparently more variation in this genus than in *Hymenophyllum*, it is highly desirable that knowledge of the development of the gametophyte should be extended to cover many more species. Information is so fragmentary that we do not know how common is the 3-celled type of germination, or its relation to the ribbon-like thallus or the mixed type in which both filaments and expansions are found. We do not know whether the pure filamentous type of gametophyte is correlated with the 4-celled type of germination, as seems probable; or even whether there is a pure filamentous type, although that seems to be the case in *T.*

*auriculatum*,<sup>\*</sup> *T. bilabiatum*, *T. maximum*, and probably in a majority of the species of *Trichomanes*. However, as is the case of *T. sinuosum* (14), the production of sex organs by the filamentous stage does not preclude the possibility of development of a plate.

A comparison of the structure of the gametophyte of some of the oriental species of *Trichomanes* with the position of the species in COPELAND'S classification may be of interest, not because it affords adequate basis for any conclusions but because of suggestions of lines of investigation. Germination stages are known for representatives of only six of COPELAND'S fifteen groups. Certain points, however, seem to be worth noting. *Cardiomanes reniforme* (Forst.) Pr. (*Trichomanes reniforme* Forst.) agrees with *Hymenophyllum* in germination and in the early formation of a plate stage; the mature stage is not known. COPELAND (7, p. 270) says of this species, "Construing *Trichomanes* in the broadest sense, this is the only really isolated species in the Old World." In his group 1, *Pyxidifera*, both types of germination have been reported: the 3-celled type in *T. draytonianum* (5) and the 4-celled in *T. schmidianum* (18, 23). Two species have been described as filamentous at maturity: *T. pyxidifera* (1) and *T. colensoi* (16). *T. diffusum*, in group 4, *Gonocormus*, has the 4-celled type of germination (12). In group 5, *Microtrichomanes*, the three species for which the germination is known belong to the *Hymenophyllum* type: *T. ampliatus* (*T. digitatum* Sw.) (18), *T. palmatifidum* (12), and *T. nitidulum*. HOLLOWAY (15) states that *T. lyallii* has early plate development. COPELAND (8, p. 165) in his discussion of *Sphaerocionium*, his eighth sub-genus of *Hymenophyllum*, says, "the whole of this group, *Microtrichomanes*, as I used the term (not as PRANTL used it), belongs rather in *Hymenophyllum*, as the term is used here." *T. humile*, in group 6, *Crepidium*, has the 4-celled type of germination (18); and *T. bilabiatum*, of group 7, *Taschneria*, has the same type. Group 9, *Scandentia*, includes three species for which germination is known: *T. speciosum* (*T. radicans* Sw.) (19), *T. auriculatum*, and *T. maximum*. This group has the modified type of germination, the 4-celled type which tends to lose its sharply defined character. In germination this group seems to be farther removed from the central type than any for which there are records.

\* When the cultures of *T. auriculatum* were two and a half years old, a few expansions three to five cells wide were found at the tips of a few filamentous branches.

Obviously much more information is needed about germination, development, and mature stages before we are in a position to estimate the value of the contribution of the gametophyte to a phylogenetic classification of the family. Even so plastic and variable a structure as the gametophyte has certain well defined characters which may be of value in the working out of groups. In a family which has presented such difficulties in classification as those noted by COPELAND, a gametophyte of so pronounced a character may well be the basis of important contributions.

### Summary

1. Germination stages are given for seven species of *Hymenophyllum*: *H. acanthoides*, *H. blumeianum*, *H. fuscum*, *H. holochilum*, *H. junghuhnii*, *H. kurzii*, and *H. javanicum*. The spores germinated while still in the sporangium with the formation of a cross wall followed by a second wall meeting the first in the center of the spore. The walls separating the three cells lie under the tripartite ridges of the spore coat. Occasionally the second wall is delayed until after discharge of the spores, or it may be suppressed.

2. After rupture of the spore coat the young gametophyte assumes the form of a triangular cushion, with the three tips growing in the direction of the longitudinal axes of the valves of the spore coat.

3. Secondary walls arise in sequence, cutting off a small cell from each tip. Development is usually symmetrical up to the 6-celled stage, but the next stages are more variable. In *H. acanthoides*, *H. blumeianum*, *H. holochilum*, and *H. kurzii* growth of one tip results in a plate; growth of the other two is checked sooner or later by rhizoid formation. The prothallus develops as a branching ribbon-like structure, which at one year may have branches 9-12 mm. long. In *H. fuscum* and *H. javanicum* growth is much slower; the later growth is filamentous in form with occasional longitudinal divisions but without plate formation.

4. Rhizoid development is belated in *Hymenophyllum*; the first rhizoid usually appears when there are six to eight cells in the gametophyte. The rhizoids have heavy brown walls; they may arise from any cell of a filament, but arise only from marginal cells of the ribbon-like thallus. Branching rhizoids are common, particularly near the

base of the gametophyte; frequently two rhizoids grow from one cell.

5. The gametophytes of *H. holochilum* and *H. kurzii* reached maturity in twenty months, bearing both antheridia and archegonia. *H. blumeanum* did not produce archegonia until nearly four years old.

6. Germination in *Trichomanes nitidulum* is like that in *Hymenophyllum*, with the spore dividing into three cells. Subsequent stages were less regular than in *Hymenophyllum* and there was no plate formation. The gametophytes remained filamentous, with longitudinal divisions in a few cells or sections of a filament.

7. In *T. auriculatum* and *T. bilabiatum*, after rupture of the spore coat and before any divisions have occurred, the spore assumes the form of a triangular cushion. The first walls cut off the three tips, leaving an undivided central cell. If growth is slow, as in the case of spores which fall into the indusium and germinate there, the 4-celled stage is symmetrical; but if conditions for growth are favorable, the form is more irregular, particularly in *T. auriculatum* in which the first tip cut off usually forms a rhizoid in which growth is relatively rapid. The wall cutting off the third tip is frequently delayed, especially in *T. auriculatum*.

8. Germination in *T. maximum* may conform to the 4-celled type, but it is usually less regular. There is a tendency toward suppression of one of the tips, or sometimes merely a delay in wall formation in one tip. The gametophyte may then develop as a straight or a lopsided filament, rather than as a triradiate structure. Growth and development in *T. maximum* are extremely slow.

9. The mature gametophytes of *T. auriculatum* and *T. bilabiatum* are of the branching filamentous type without longitudinal divisions, forming a "turf" in compact cultures. They attained maturity, producing antheridia and archegonia, in nine to ten months. The gametophyte of *T. maximum*, although developing much more slowly, was similar in form and habit. Antheridia were formed on gametophytes one year old.

10. A variation from the usual type of germination in *Hymenophyllum* was found occasionally in several species. It suggests how the two types of germination in the family may be related. Reasons

are given for looking upon the *Hymenophyllum* type as the older. Gametophytes of *Hymenophyllum* are found occasionally in which only one primary wall is formed at germination; the second is suppressed. The 4-celled type found in most species of *Trichomanes* might have arisen from the type found in *Hymenophyllum* (and a few species of *Trichomanes*) by the suppression of both of the primary walls, leaving the central part of the spore undivided. In the latter type of germination the first walls to appear in the spore correspond to the secondary walls which appear in the tips of the triangular gametophyte of *Hymenophyllum*.

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# ABSORPTION OF SOIL MOISTURE BY MAIZE ROOTS

CHARLES HOMER DAVIS

(WITH THREE FIGURES)

## Introduction

Little is known of the mechanics of water absorption from the soil by the roots of plants. ROSENE (7) has recently summarized the data on water absorption by different root zones. WEAVER (8) found that root hairs soon died in dry soil. POPESCO (5) noted that dead root hairs could be active in absorption. ALDRICH, WORK, and LEWIS (1) found a high correlation between the concentration of fine roots and the amount of water absorbed in the previous season. They observed that the growth of pear fruits stopped well before the soil water was reduced to the wilting coefficient, and also that the leaves wilted before the wilting point of the soil moisture was reached. LOOMIS (4) observed that maize plants stopped growth under conditions of high transpiration although the moisture present in the soil was nearly optimum. ALDRICH, WORK, and LEWIS pointed out the possibility that wilting of the leaves of pear trees may have been caused by the drying of the soil below the wilting point within a short distance of the small roots, while large samples necessary for moisture determinations did not show a critically low moisture content. The present investigation, started in 1937, was designed to determine the absorption of water from soil in which root development could be observed.

## Investigation

### RELATION OF ROOT POSITION TO MOISTURE ABSORPTION

Maize was planted at one end of a wooden box in which one side had been replaced with glass, through which the roots could be observed as they grew outward and downward from the plant (fig. 1). Rate of growth and distribution of the roots were studied daily by removing the black cardboard, which covered the glass side, and observing the roots which could be seen through the glass. Moisture absorption was measured by daily readings of RICHARDS' soil-mois-

ture tensiometers (6) set at 4-inch intervals in the box, 6 inches below the surface and halfway between the back and front of the box. Tensiometers measure the surface tension of the water directly with a mercury manometer, which is connected to the soil moisture through the walls of a porous clay cup by an air-free water system. Since the mercury is in a capillary tube and its weight resists the loss

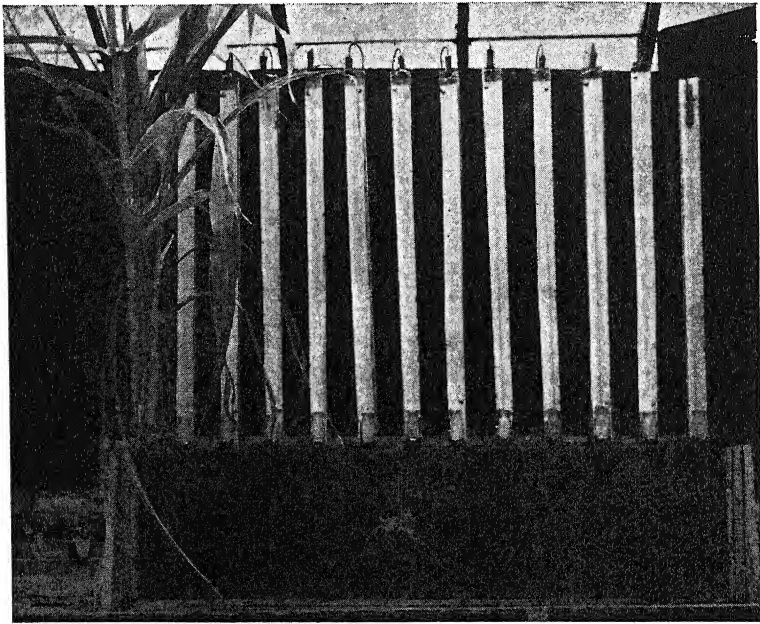


FIG. 1.—Box used in the greenhouse at Ames, showing arrangement of plants and tensiometers.

of water to the soil, only a small quantity of water is lost to the soil from the tensiometer. The mercury manometer is dependent upon air pressure, so the limit of tension measured by the tensiometer is one atmosphere. The instruments used in these experiments failed to record accurately above 600 mm. of mercury. Soil moisture percentages below the range of the tensiometers were determined by sampling with a sharpened steel tube.

Figures 2 and 3 give the moisture levels, reciprocal of tension reading, for the alternate days during two drying periods. During the first period the roots had extended as shown at the top of figure 2

on the dates indicated. The roots reached the end of the box April 6 and thereafter appeared to be uniformly distributed throughout the

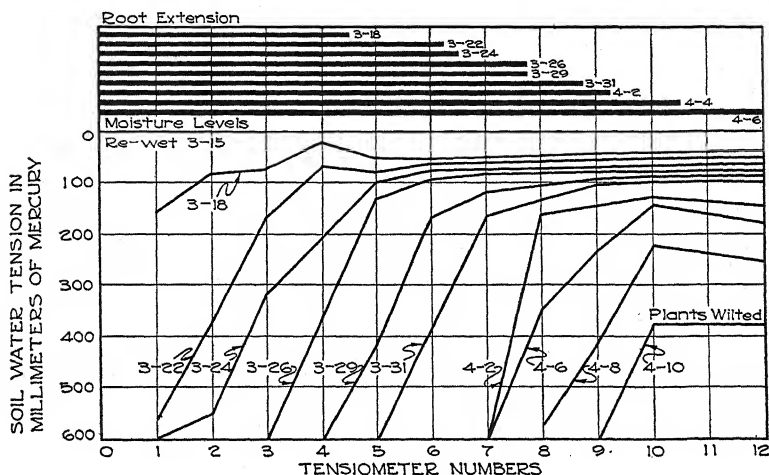


FIG. 2.—Root extension (top) and moisture absorption tensions at distances of 4-48 inches from group of corn plants.

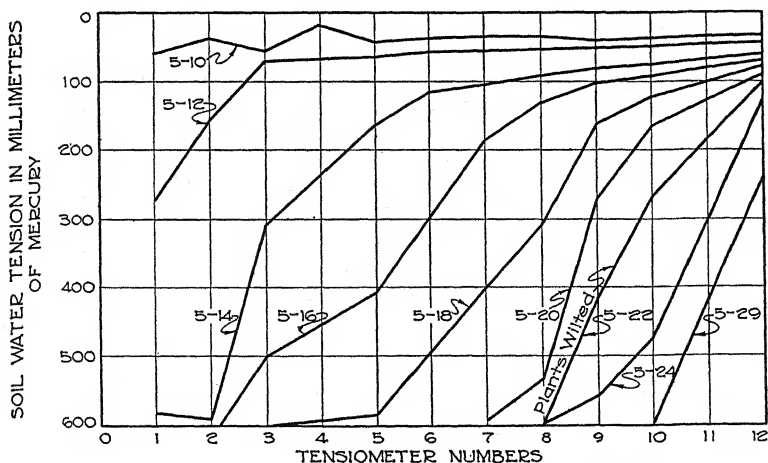


FIG. 3.—Moisture absorption tensions in a box uniformly filled with roots from plants at the left. Tensiometers spaced 4 inches apart.

soil. The soil was watered on March 15 and seven days later the soil near the plants had been dried almost to the limit of the tensiometer.

eter range. At the same time the roots had extended past tensiometer no. 6, 24 inches from the plant, but appreciable moisture absorption took place no farther than 12 inches from the plant, as shown by the rise in tensiometer no. 3. Progressive absorption is shown for the successive periods. On March 31, when the roots were 7 inches beyond tensiometer no. 7, appreciable moisture absorption was taking place near that tensiometer. Two days later the soil near no. 7 was too dry to measure but appreciable moisture absorption was taking place near tensiometer no. 8. Roots had extended 6 inches past no. 9 on April 2 but that tensiometer did not show appreciable water absorption from the soil until April 6, when the roots had reached the end of the box. The slow increase in tension shown by the tensiometers not in the root area was probably due to air drying, although the soil was covered with a 3:2 paraffin-vaseline mixture to reduce this loss. On April 10 roots were established in moist soil in the end of the box 4 feet from the plants, yet the plants were permanently wilted under greenhouse conditions.

The wilting percentage of the soil was determined with maize from the same seed lot. The average of twenty-five determinations was 9.58 per cent, and the range was from 8.28 when the plants in a pot were severely wilted to 11.60 per cent soil moisture in a pot in which the plants were barely wilted but remained wilted overnight in a moist chamber. The soil in the box on April 16, as shown in table 1, was below the determined wilting percentage 2 feet from the plants and the plants remained wilted overnight; yet 3 feet from the plants the soil moisture was above the wilting percentage and half the soil available to the root system contained available moisture.

The soil was then re-wet and the moisture was again absorbed, first near the plants and later farther from them. Root hairs were more numerous at all times on the root tips farther from the plants, and in relatively moist soil, than on the roots near the plants in soil which was dried more frequently. All the roots had many small branches, on a few of which root hairs developed. Those fine roots with hairs which appeared on the glass front soon lost their hairs, and root hairs were never observed on many of the small rootlets.

After April 26 the soil as seen through the glass front was uniformly filled with roots and the moisture absorptions shown in ta-

TABLE 1

MOISTURE PERCENTAGES BY DATE AND POSITION, AND NUMBERS OF ROOTS  
 APPEARING AGAINST EACH 4 INCHES OF GLASS FRONT OF THE BOX  
 EXPERIMENT AT AMES. WILTING PERCENTAGE OF SOIL, 9.6

DATE	POSITION OF SAMPLES AND ROOTS IN INCHES FROM PLANTS												INCHES DEPTH	NOTES
	4	8	12	16	20	24	28	32	36	40	44			
March 11.....	...	...	...	14	...	...	...	...	15	...	...	1-3	Plants wilted	
	...	...	...	13	...	...	...	...	16	...	...	3-6		
	...	...	...	14	...	...	...	...	14	...	...	6-10		
No. roots.....	16	21	14	9	...	...	...	...	...	...	...	...		
March 12.....	...	19	...	...	...	...	...	...	19	...	...	1-3	Re-wet after sampling	
	...	15	...	...	...	...	...	...	15	...	...	3-6		
	...	12	...	...	...	...	...	...	15	...	...	6-10		
No. roots.....	16	21	16	9	...	...	...	...	...	...	...	...		
March 17.....	...	...	21	...	...	...	...	...	...	25	...	1-3		
	...	...	23	...	...	...	...	...	...	23	...	3-6		
	...	...	20	...	...	...	...	...	...	26	...	6-10		
No. roots.....	16	21	16	14	...	...	...	...	...	...	...	...		
March 29.....	...	13	...	...	...	19	...	...	...	25	...	1-3	Re-wet April 10	
	...	11	...	...	...	19	...	...	...	22	...	3-6		
	...	10	...	...	...	19	...	...	...	22	...	6-10		
No. roots.....	16	22	16	19	5	9	5	2	1	...	...	...		
April 16.....	...	...	...	8	...	8	...	...	13	...	...	1-3	Re-wet after sampling	
	...	...	...	8	...	7	...	...	11	...	...	3-6		
	...	...	...	7	...	8	...	...	9	...	...	6-10		
No. roots.....	16	22	16	23	14	23	16	9	7	2	2	.....		
April 18.....	...	...	...	25	...	...	30	...	27	...	...	1-3		
	...	...	...	21	...	...	23	...	24	...	...	3-6		
	...	...	...	9	...	...	22	...	22	...	...	6-10		
No. roots.....	16	22	16	23	14	23	16	12	7	5	2	.....		
April 26.....	10	...	...	13	...	...	15	...	19	...	...	1-3	Wilted May 5	
	10	...	...	11	...	...	14	...	17	...	...	3-6		
	10	...	...	10	...	...	14	...	16	...	...	6-10		
No. roots.....	16	22	16	23	14	23	16	14	13	17	14	.....		
May 7.....	...	...	7	...	...	...	8	...	11	...	...	1-3	Re-wet after sampling	
	...	...	7	...	...	...	8	...	9	...	...	3-6		
	...	...	7	...	...	...	7	...	9	...	...	6-10		

TABLE 1—*Continued*

DATE	POSITION OF SAMPLES AND ROOTS IN INCHES FROM PLANTS											INCHES DEPTH	NOTES
	4	8	12	16	20	24	28	32	36	40	44		
May 10.....	...	...	22	...	...	...	21	...	...	22	...	1-3	
	...	...	22	...	...	...	22	...	...	23	...	3-6	
	...	...	23	...	...	...	23	...	...	24	...	6-10	
May 15.....	9	...	...	13	...	...	...	18	...	...	...	1-3	
	9	...	...	13	...	...	...	18	...	...	...	3-6	
	9	...	...	11	...	...	...	19	...	...	...	6-10	
May 22.....	...	8	...	...	...	...	...	...	14	...	18	1-3	Wilted at noon
	...	8	...	...	...	...	...	...	13	...	18	3-6	
	...	8	...	...	...	...	...	...	12	...	18	6-10	
May 29.....	...	...	7	...	...	...	8	...	...	...	16	1-3	Re-wet after sampling
	...	...	8	...	...	...	9	...	...	...	16	3-6	
	...	...	7	...	...	...	8	...	...	...	15	6-10	
June 8.....	10	...	16	...	...	...	...	...	19	...	...	1-3	
	10	...	14	...	...	...	...	...	20	...	...	3-6	
	9	...	14	...	...	...	...	...	20	...	...	6-10	

ble 1 after April 26 and in figure 3 represent absorption at different rates by apparently equal numbers of roots. Between May 10 and 14 the soil near tensiometer no. 1 was dried by water absorption to a moisture percentage below the wilting percentage, while 20 inches from the plants only a small amount of water had been absorbed. Six days later, on May 20, the soil near tensiometer no. 8, 32 inches from the plant, was almost as dry as the lower moisture range of the tensiometer, but only small amounts of water were being absorbed near no. 12. The plants wilted at noon on May 22, two days later, when the soil moisture as near the plant as no. 9 and no. 10 (36 inches away) was still above the wilting percentage, and the soil moisture near no. 11 was only 2 per cent below the field capacity of the soil. Moisture near the plants was reduced to the wilting percentage on May 15, seven days before the plants showed any tendency to wilt. The box was re-wet after May 29 and on June 8 the soil near the plants was already near the wilting percentage, while 3 feet away from the plants moisture in the soil was near the field capacity.

The soil used at Ames was Clarion silt loam. Later the same ex-

periments were repeated at Tucson, Arizona, with Gila silt loam. The experiments at Tucson differed in that tensiometers were not used; one plant instead of five was left in the box; the roots were counted more accurately to include all the fine roots, and the box was slightly longer (53 inches). In the absence of tensiometers, moisture samples were taken more regularly. The percentages of moisture found are shown in table 2. The two samples for a given location in a single day were taken from the surface to 6 inches deep and from 6 inches to the bottom of the box. The observed wilting percentage of the soil used at Tucson was 6.76. On November 29 the plant wilted at noon. The percentages of soil moisture near the plant were near the wilting percentage, but 20 inches from the plant water was readily available. The plant recovered overnight. On December 7 the plant wilted at 10 A.M., when the moisture samples were taken (table 2). After the low values of 3 and 5 per cent were found, samples were taken again on December 8 to check these values. The second samples, 8 inches farther from the plant, contained only 4 and 5 per cent moisture and show that the roots near the plant absorbed water well below the determined wilting percentage, while roots farther from the plant were still in soil moistened above the wilting percentage. The plant did not recover turgor after wilting on December 7 until the soil was re-wet on December 8.

From December 8 to 21, roots 32 inches from the plant and ranging in number from sixteen to seventy absorbed water down to 13 and 11 per cent soil moisture. During the same period twenty-nine to thirty-three roots 8 inches from the plant absorbed water down to 7 and 8 per cent soil moisture, or about the wilting percentage. After each wetting the same greater absorption near the plant was observed, although similar numbers of roots were present farther from the plant.

The box was thoroughly saturated on December 24 and by December 27 the soil was dry enough to sample. On January 2 appreciable quantities of moisture had been absorbed near the plant, while 24 inches away the soil moisture was near the field percentage. After the samples of January 2 were taken, the soil was cut into 3-inch sections and the roots appearing in the cross sections were counted. The numbers of roots and the average of the numbers of roots of the

TABLE 2

MOISTURE PERCENTAGES AND ROOT DISTRIBUTION BY 4-INCH INTERVALS  
EXPERIMENT AT TUCSON. WILTING PERCENTAGE, 6.8

DATE	DISTANCE FROM PLANT IN INCHES													NOTES
	4	8	12	16	20	24	28	32	36	40	44	48	53	
November 4.....	{	5	...	...	8	...	...	...	10	...	...	...	...	Re-wet after sampling
		8	...	...	8	...	...	...	12	...	...	...	...	
No. roots.....		3	...	...	...	...	...	...	...	...	...	...	...	
November 10....	{	...	25	23	22	...	...	...	...	22	...	...	...	
		...	29	17	23	...	...	...	...	25	...	...	...	
No. roots.....		3	...	...	1	...	...	...	...	...	...	...	...	
November 19....	{	8	...	9	...	...	14	...	...	15	...	...	...	
		8	...	9	...	...	15	...	...	15	...	...	...	
No. roots.....		5	3	10	5	3	2	...	...	...	...	...	...	
November 21....	{	...	6	...	...	10	...	11	...	...	12	...	...	
		...	7	...	...	9	...	13	...	...	15	...	...	
No. roots.....		8	14	16	21	17	12	9	...	...	...	...	...	
November 29....	{	...	7	...	...	13	...	16	...	...	19	...	...	Plant wilted at noon
		...	8	...	...	9	...	17	...	...	22	...	...	
No. roots.....		12	23	33	52	50	52	24	9	...	...	...	...	
December 7.....	{	3	...	...	...	...	6	...	10	12	...	...	...	Wilted at 10 A.M.
		5	...	...	...	...	6	...	10	15	...	...	...	
No. roots.....		19	29	31	53	52	51	20	17	4	...	...	...	
December 8.....	{	...	...	4	...	...	...	6	...	...	...	...	...	Wilted, re-wet
		...	...	5	...	...	...	7	...	...	...	...	...	
December 18....	{	...	...	10	...	13	...	...	...	16	...	19	...	
		...	...	10	...	11	...	...	...	19	...	19	...	
No. roots.....		28	33	37	65	83	69	32	56	43	28	15	...	
December 21....	{	...	7	...	...	10	...	...	13	...	16	...	17	
		...	8	...	...	8	...	...	11	...	18	...	19	
No. roots.....		40	33	51	71	86	77	33	70	66	54	39	3	
December 24....	{	...	...	...	6	...	8	...	...	...	13	13	...	Wilted, re-wet
		...	...	...	6	...	7	...	...	...	12	15	...	
No. roots.....		36	34	48	58	73	58	36	69	53	59	47	10	

TABLE 2—*Continued*

DATE	DISTANCE FROM PLANT IN INCHES													NOTES
	4	8	12	16	20	24	28	32	36	40	44	48	53	
December 27....	23	...	30	...	...	...	...	29	...	...	...	34	...	Soil still flooded
	24	...	26	...	...	...	...	30	...	...	...	34	...	
No. roots.....	41	36	49	57	75	60	39	64	64	68	52	28	30	
January 1.....	...	17	...	19	...	...	...	19	...	26	...	...	25	
	...	16	...	19	...	...	...	21	...	25	...	...	26	
No. roots.....	37	33	45	61	80	64	39	50	60	71	70	52	28	
January 2.....	11	...	16	...	17	22	...	22	...	20	22	24	25	
	15	...	17	...	...	20	...	23	...	...	25	25	...	

three adjacent 1×10-inch areas on the glass front are recorded in table 3. The correlation coefficient of 0.81 is positive and significant, showing that the counts made on the glass front were a reasonably good estimate of the total numbers of roots in the box.

TABLE 3

CORRELATION BETWEEN ROOTS IN CROSS SECTION OF SOIL AND IN ADJACENT SURFACE EXPOSED THROUGH A GLASS WALL

AREA	INCHES FROM PLANT															
	3	6	9	12	15	18	21	24	27	30	33	36	39	42	45	48
Roots in soil section..	54	50	47	41	49	58	67	56	62	48	66	60	64	62	59	40
Roots in glass front..	9	14	12	11	14	17	20	14	10	15	13	16	20	21	14	9
Correlation..... $r = +0.81$																

#### DEPENDENCE OF GROWTH UPON SOIL MOISTURE

The effect of moisture supply on the growth of young maize plants was studied in 2-gallon pots in which the factor of distance between plant and soil moisture was minimized. When the plants were approximately 40 mm. high they were thinned to ten in each of four pots. The percentages of moisture in the soil were calculated from

the gross weights of the pots and wax seal, and the known weights of the dry soil they contained. Growth of the plants was estimated by the total increases in height of the tallest leaves on all the plants in a pot and calculated as growth in millimeters per pot per hour. During the 41 days of growth from which the data of table 4 were calculated there was no evidence of a logarithmic growth rate. The pronounced effects on growth rate caused by the fluctuations in soil moisture completely obscured any logarithmic relationships.

The first line of table 4 shows that the total growth in the 24 hours just preceding the measurement on pot 1 averaged 0.88 mm. an hour; at the time of making the growth measurements the pot was weighed, and the soil moisture percentage calculated from the gross weight was 16.96. During the next period the ten plants grew at the rate of 1.52 mm. per hour and the soil moisture percentage at the end of the period had dropped to 14.61. During the third period the growth rate was reduced to 0.37 mm. per hour and the soil moisture to 10.48 per cent. During the next period the growth was negative, a loss of 6 mm. in twenty hours, and the soil moisture was reduced to 9.63 per cent. Negative growths were caused by necrosis of the tender tips as the soil became dry. After the plants had wilted or stopped growth the pots were re-wet to the capillary capacity of the soil and the measurements continued. In many cases several days were required for the young growing leaf to reach the height attained by an older leaf which had stopped growth during the dry days. These tardy tips just after wetting resulted in lower rates of increase than would have resulted from measurements of the growing tip. The intervals between measurements were not so regular as desired and in some cases the rates of increase were calculated for as long as three days. The final moisture percentages after the longer periods are too low an estimate of the soil moisture conditions under which growth took place. Negative growths were not used in the calculation of the correlation coefficient because they were influenced greatly by a single tender tip at the time the pot became dry. The correlation coefficient of 0.53 is positive and significant, and includes discrepancies in time of measurement and soil moisture determinations, which usually were too low, and excludes negative rates of growth. The numerical value of the coefficient, but not the significance, could be increased by ex-

TABLE 4

CORRELATION OF SOIL MOISTURE PERCENTAGES WITH TOTAL INCREASES IN PLANT HEIGHT IN MILLIMETERS PER POT PER HOUR MEASUREMENTS WHICH SHOWED NO GROWTH OMITTED. WILTING PERCENTAGE OF SOIL, 6.8

PERCENTAGE MOISTURE	RATE OF GROWTH	PERCENTAGE MOISTURE	RATE OF GROWTH
Pot I		Pot III	
16.96.....	0.88	15.86.....	1.04
14.61.....	1.52	13.21.....	0.87
10.48.....	0.37	10.92.....	1.78
21.24.....	7.71	10.57.....	1.31
20.63.....	6.79	18.66.....	9.38
15.86.....	5.35	12.42.....	1.60
11.97.....	3.42	23.67.....	3.62
21.84.....	3.00	13.24.....	3.08
16.58.....	3.92	10.81.....	1.22
11.41.....	1.95	17.61.....	4.33
10.67.....	2.54	13.17.....	0.26
22.48.....	3.73	9.50.....	0.37
16.83.....	1.45	19.13.....	2.30
10.76.....	2.50	12.13.....	0.14
16.74.....	1.96	.....	.....
12.81.....	0.37	.....	.....
10.70.....	0.17	.....	.....
13.67.....	1.69	.....	.....
16.81.....	4.07	.....	.....
12.08.....	1.83	.....	.....
10.25.....	0.43	.....	.....
Pot II		Pot IV	
19.42.....	1.37	17.80.....	0.87
16.73.....	0.82	15.09.....	1.11
11.52.....	0.46	10.86.....	0.27
10.37.....	0.18	9.74.....	0.29
23.40.....	7.29	23.50.....	8.21
22.65.....	4.71	18.01.....	5.85
17.55.....	10.30	13.25.....	7.79
12.99.....	6.92	16.38.....	2.42
17.24.....	3.40	11.07.....	1.91
10.93.....	3.84	10.73.....	2.02
10.08.....	3.41	23.84.....	2.78
24.45.....	2.78	17.83.....	2.43
18.62.....	7.62	11.19.....	2.17
10.70.....	2.54	18.33.....	4.19
18.48.....	3.19	13.70.....	2.26
13.34.....	2.67	11.20.....	0.13
10.84.....	0.09	18.24.....	0.67
14.71.....	1.55	18.41.....	6.45
18.98.....	3.72	14.00.....	4.00
10.24.....	0.54	10.67.....	0.07

Correlation.....  $r = +0.53$

cluding some of the rates taken for longer periods and by using average soil moisture percentages, or by using percentage of available moisture rather than the directly measured soil moisture.

Under the conditions of this experiment the growth of young maize plants was influenced directly by the amount of moisture in the soil from the capillary capacity, 23 per cent, to 9.5 per cent. Plants ceased to grow or decreased in total leaf height when the soil moisture percentage was below 9.5. Table 5 shows the average moisture percentages at the ends of the periods during which growth of the plants ceased from lack of moisture.

TABLE 5  
MOISTURE PERCENTAGES IN POTS AT TIME GROWTH STOPPED

Pot I	Pot II	Pot III	Pot IV
10.06	9.86	9.74	9.38
10.76	10.02	9.98	11.74
9.57	9.57	9.50	9.94
9.78	9.86	11.30	10.07
Average.....			9.88
Wilting percentage.....			6.76
Average available moisture when growth stopped.....			3.12

The pots were watered when growth stopped. When growth stopped before wilting occurred, the cessation of growth does not represent observable wilting in any of the plants during any part of the day observed. The soil moisture percentages when growth ceased ranged from 11.74 to 9.38. The average of the sixteen determinations was 9.88 per cent. The wilting percentage, determined as the moisture to which these plants were able to reduce the soil in the pots, was 6.76, and the wilting coefficient calculated from the moisture equivalent was 9.1 per cent soil moisture. Growth of the plants stopped when 3 per cent of soil moisture as measured by the wilting percentage and 0.75 per cent of soil moisture as measured by the wilting coefficient were available.

### Discussion

The selective absorption of water near the plant, when similar numbers of roots of the same plant were in soil of higher moisture content farther from the plant, indicates a moisture absorption gradient in the roots. The roots near the plant absorbed water from soil at or below the wilting percentage, while the same root or other roots 2-4 feet away were absorbing moisture at a level halfway between the wilting percentage and the field percentage. After the soil near the plant was reduced below the wilting percentage, the roots in soil moistened 4-5 per cent above the wilting percentage but 4 feet from the plant did not obtain enough water to prevent permanent wilting. There is thus no evidence for a "build up" of moisture in the soil near the plant, such as was shown by BREAZEALE (2), even when the roots 3-4 feet away were in soil moisture at or near the capillary capacity of the soil.

The differential rates of absorption near and away from the plant introduce a factor of distance into the wilting percentage of the soil. Should larger volumes of soil be used in determining the wilting percentage, as has been done in the field by ALDRICH, WORK, and LEWIS (1), BURR (3), and others, the field moisture percentage at wilting or maturation of a crop might be either above or below the wilting percentage determined with the same plants in a small pot. The availability of water in this experiment was not measured alone by the force with which the soil held the water, or the soil moisture percentage, but also by the distance between the moisture and the plant.

Growth of corn leaves was directly related to the amount of available moisture in the soil. Corn leaves grew less rapidly as the moisture supply was reduced. The availability of water for growth thus appeared to be less as the soil water was reduced only 2 or 3 per cent below the capillary capacity of the soil, and apparently growth no longer took place when 3 per cent of moisture was still available for the life of the plant. Plants in the large glass-front boxes did not stop growth after temporary wilting in the afternoons when sufficient moisture was available for them to regain turgor under cooler and more humid conditions; but even though moisture was available 3-4 feet from the plants, insufficient moisture was absorbed to

maintain turgor or growth when the soil near the plant was dry. The plants observed in these experiments appeared to grow more rapidly when adequate water was supplied to the leaves than when the water supply was reduced, as the moisture percentage near the plant was lowered. Measurements of ALDRICH, WORK, and LEWIS (1) on pear fruits and their observations on the leaves of the same trees indicate that the fruits and the leaves were not supplied equally with moisture as the water supply decreased. Their data on fruit growth and the observations of corn just described indicate the dynamic and complex nature of the relation between soil moisture and plant growth and demonstrate the need for more information on the problem.

### Summary

1. Observations of roots of young maize plants in a glass-front box were reasonably good estimates of the numbers of roots in a narrow box in the greenhouse.

2. Roots of established plants absorbed water from the soil more rapidly near the plant than at a distance of 3 or 4 feet. Roots of growing plants extracted water below the wilting percentage in soil near the plant when similar numbers of roots were present in moisture above the wilting percentage 4 feet away.

3. The water 4 feet from the plant was eventually absorbed after the soil near the plant was dried below the wilting percentage.

4. Growth of young plants under the conditions of these experiments was slowed by decreasing soil moisture and was stopped when the soil moisture percentage was still above the wilting percentage and the calculated wilting coefficient.

5. The absorption of water by roots is apparently a dynamic activity depending on the quantity of water in the soil and the distance between the water and the transpiring tops of the plants.

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# FLORAL INITIATION IN BILOXI SOYBEANS AS INFLUENCED BY AGE AND POSITION OF LEAF RECEIVING PHOTOPERIODIC TREATMENT

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## Introduction

It has been demonstrated with various plants (1, 2, 3, 4, 5) that the photoperiodic stimulus causing initiation of flowers is received by the leaves. Young leaves of soybeans when subjected to short photoperiods have been found to be very effective in causing such initiation. This fact may be due to the age of the leaf or to its proximity to the terminal meristem. The purpose of this investigation was to determine the relative influence that these factors play in controlling photoperiodic induction.

## Material and methods

Biloxi soybeans were grown in the greenhouse on daily photoperiods of 16 or more hours until they reached the size or age desired. In certain experiments photoperiodic treatments were begun as soon as the primary leaves were fully expanded; in others the treatments were not started until the plants had produced several compound leaves. At the beginning of the various experiments, dissections were made of representative plants to make certain that none had yet initiated flower primordia. Control plants grown continuously on long photoperiod were included in each experiment.

The effectiveness of each photoperiodic treatment was judged by the number of plants per lot that initiated flower primordia and by the number and location of those so formed. In several of the experiments these observations were made in the terminal bud of the main axis; in others this bud was removed and the observations were made in the terminal buds of branches developed from certain axillary buds. Thus in both types of experiments observations were made in

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terminal buds; in one case they were terminals of the main axis and in the other they were terminals of side branches. In both cases flower primordia were also formed in axillary buds below the terminals. The data obtained from these axillary buds, however, contribute little that is not shown in the terminals. This makes it unnecessary in many studies of floral initiation to obtain data from both kinds of buds.

### Experimental results

The purpose of the first experiment was to study the relative photoperiodic effectiveness of certain individual leaves, parts of leaves, and combinations of two or more leaves. All leaves or parts of leaves except those required in the particular treatment were removed. The plants, defoliated in a variety of ways, were then subjected to 8-hour photoperiods. One-third of the plants of each group received two short photoperiods, another third received four short photoperiods, and the remaining plants were subjected to six short photoperiods. Photoperiodic treatments of all lots were started simultaneously. When these were completed, the plants were returned to photoperiods of 16 or more hours and continued under these conditions until 3 weeks after the beginning of photoperiodic treatments. All plants were then harvested and dissected.

The plants were selected for uniformity of size from a large population. Experimental treatments were started 24 days after the seed was planted. At that time the plants were expanding their fourth compound leaves, and special care was given to the selection of plants whose fourth leaves were of nearly the same size and stage of development.

The total number of plants per lot that had initiated flower primordia was determined and the number and location of flower buds on these plants were recorded. Counts were also made of the nodes in the main axis of each plant.

The third compound leaf on these plants was found to be more effective than any other in causing initiation of flower primordia (table 1). Floral initiation occurred following two short photoperiods in thirty-four of the forty plants in lots 6, 7, 8, and 14, in which lots part or all of the third leaf was present. In the remaining nine partially defoliated lots, none of which involved the third leaf,

only four plants in ninety initiated flower buds. All plants in the undefoliated control (lot 1) bore flower primordia following two short photoperiods.

When longer induction treatments of 4 and 6 short days were given, all plants having a third compound leaf initiated flower

TABLE 1

EFFECT OF VARIOUS METHODS OF DEFOLIATION ON FLORAL INITIATION IN  
BILOXI SOYBEANS SUBJECTED TO TWO, FOUR, AND SIX  
PHOTOPERIODS OF 8 HOURS

LOT NO.	LEAVES PRESENT ON PLANTS DURING INDUCTION TREATMENTS	TOTAL PLANTS PER LOT OF 10 BEARING FLOWER PRIMORDIA AFTER VARIOUS PERIODS OF INDUCTION			TOTAL BUDS BEARING FLOWER PRIMORDIA ON MAIN AXIS PER LOT OF 10 AFTER VARIOUS PERIODS OF INDUCTION		
		2 DAYS	4 DAYS	6 DAYS	2 DAYS	4 DAYS	6 DAYS
1.....	All	10	10	10	12	36	54
2.....	2 primaries	0	3	9	0	4	15
3.....	1 primary	0	3	7	0	3	9
4.....	First compound	0	10	10	0	17	27
5.....	Second compound	0	10	10	0	24	34
6.....	Third compound	9	10	10	10	42	56
7.....	Third compound (center leaflet only)	7	10	10	8	41	48
8.....	Third compound (2 lateral leaflets only)	9	10	10	15	33	54
9.....	Fourth compound	2	10	10	2	29	45
10.....	Fourth compound and 1 primary	1	3	8	3	9	41
11.....	Fourth compound and 2 primaries	0	7	10	0	24	34
12.....	Fourth and first compound	0	10	10	0	29	42
13.....	Fourth and second compound	1	10	10	1	19	45
14.....	Fourth and third compound	9	10	10	11	38	53

primordia. Other lots showed progressive increase with these longer induction treatments, and in the 6-day treatment all plants in most lots and nearly all in the remaining ones formed flower primordia.

Although most plants receiving 6-day induction treatments initiated flower primordia, there was considerable difference between lots as to the number formed. Those in which the third compound leaf was present—either alone, in part, or in combination with other leaves—were equal or superior to all others in this respect.

It is recognized that the experimental treatments involved rather drastic defoliations which may have interfered with differentiation of new structures. Since flower primordia are formed from newly differentiated structures, it is obvious that treatments that stop or retard differentiation may interfere with floral initiation; therefore this possibility must be considered in interpreting the data. A measure of the differences in differentiation of new structures induced on defoliated plants was obtained by determining the total nodes per plant.

Since total nodes per plant in eight of the defoliated lots differed only slightly from the number of those of the undefoliated lot, the data are not presented in detail. The differences among these eight lots were not significant, even at odds as low as 19:1. In five lots, however, there was an appreciable reduction in nodes. These lots were those involving primary leaves, fourth compound leaves, or combinations of these leaves only. The differences in total nodes between these lots and the undefoliated control were in no case greater than two nodes, but they were statistically significant at odds of 99:1.

Analysis of the data on total nodes per plant and total flower primordia initiated per plant seems to show that the differences in formation of flower primordia are caused chiefly by differences in photoperiodic effectiveness of the leaves concerned. Plants having only a fourth compound leaf initiated 4.5 flower buds per plant following a 6-day induction treatment, while those with only a first compound leaf initiated 2.7 flower buds per plant when similarly treated. Removal of all but the fourth compound leaf appreciably reduced the number of nodes per plant, but removal of all but the first compound leaf had little effect. The reduction below the control in the former case was 1.2 nodes and in the latter 0.3 nodes per plant. Although neither of these differences is large, the first is statistically significant at odds of 99:1. In the case of these two lots the plants that formed fewer new structures actually produced a greater number of flower buds than did those of the other lot. The fourth leaf apparently produced a stronger flower-forming stimulus than did the first.

In a second experiment the plants were just expanding the first

compound leaf at the time photoperiodic treatments were begun. As in the former experiment, photoperiodic treatments were applied for 2, 4, and 6 short days. Methods of defoliation are indicated in table 2. Cotyledons were still present on these plants and were not removed.

Where half leaves were left on the plants the cut was made parallel to the midrib, leaving about 1 mm. of leaf blade along one side of the

TABLE 2

EFFECT OF VARIOUS METHODS OF DEFOLIATION ON FLORAL INITIATION IN  
BILOXI SOYBEANS SUBJECTED TO TWO, FOUR, AND SIX  
PHOTOPERIODS OF 8 HOURS

LOT NO.	LEAVES PRESENT ON PLANT DURING INDUCTION TREATMENT	TOTAL NODES PER PLANT AFTER VARIOUS PERIODS OF INDUCTION			TOTAL FLOWER-BEARING PLANTS PER LOT OF 10 AFTER VARIOUS PERIODS OF INDUCTION			TOTAL BUDS ON MAIN AXIS BEARING FLOWER PRIMORDIA PER LOT OF 10 AFTER VARIOUS PERIODS OF INDUCTION		
		2 DAYS	4 DAYS	6 DAYS	2 DAYS	4 DAYS	6 DAYS	2 DAYS	4 DAYS	6 DAYS
1....	All leaves	19.0	19.4	19.4	1	10	10	2	37	49
2....	Two primary leaves	19.1	19.0	19.1	0	9	10	0	10	29
3....	One primary leaf	18.7	18.9	18.9	0	6	10	0	6	25
4....	Half primary leaf	17.9	18.5	18.8	0	3	10	0	5	19
5....	One-quarter primary leaf	17.4	18.1	18.0	0	0	8	0	0	8
6....	First compound leaf	17.4	18.0	19.0	0	10	10	0	21	54
7....	No leaves	16.6	16.3	17.6	0	0	0	0	0	0

midrib. Quarter leaves were made by cutting half leaves transversely about midway of the midrib.

Only one plant in all the lots initiated flowers in response to two short photoperiods. Following four short photoperiods, however, all undefoliated plants and all those having only the expanding first compound leaf initiated flower buds. With six short photoperiods nearly all plants formed flower primordia except those in which all leaves were removed.

In this experiment the two primary leaves were approximately half as effective as the expanding first compound leaf in causing floral initiation (table 2). With only the two primary leaves present, twenty-nine flower buds were formed on ten plants in response to a

6-day induction treatment. With only the first compound leaf present, fifty-four flower buds were formed in a lot of ten plants as a result of similar treatment. Plants in these two lots differentiated about the same number of total nodes, but since one lot initiated about half as many flower buds as the other, the data suggest that the leaves of this lot supplied less flower-forming stimulus than those of the other. When the area of the primary leaves was reduced, as in lots 3, 4, 5, and 7, each decrease in area resulted in a decrease in the total number of flower primordia formed per lot and the number of nodes formed per plant. The data seem to indicate that the decrease in flower bud formation resulted from a decreased flower-forming stimulus, rather than from the reduction in the total nodes formed.

Plants of this experiment having only first compound leaves initiated about the same number of flower primordia in response to 6-day induction as plants having all of their leaves (table 2). Apparently the flower-forming stimulus supplied by this one leaf was sufficient to cause the formation of flower primordia in all new buds formed by the plant, and the additional stimulus supplied by the primary leaves when they also were present caused no further increase.

In a third experiment the plants were expanding their third compound leaves when photoperiodic treatments were started and were thus intermediate in size between those of the other two experiments. The second compound leaf of these plants was in about the same stage of maturity as the third compound leaf of experiment 1 and was slightly further advanced than the first compound leaf of experiment 2. The data (table 3) show that when the second compound leaf alone was present on the plant, as many flower buds were formed per lot as when all the leaves were present. When the two lateral leaflets of this leaf were removed, the terminal leaflet alone was still more effective in causing floral initiation than any other leaf on the plants. As a result of a 6-day induction treatment, plants with only their primary leaves initiated thirty-one flower buds per lot; plants with only the first compound leaf initiated fifty-one flower buds per lot; plants with only the second compound leaf initiated sixty-nine flower buds per lot; and those with only the third compound leaf initiated forty-five flower buds per lot. Thus the ability of individual leaves to produce a flower-forming stimulus

increased until the leaf was fully expanded and then decreased. A similar trend was evident in the data of experiment 1.

Although the preceding experiments suggest that the leaves of Biloxi soybeans decrease in their capacity to cause floral initiation as they increase in age, it must be borne in mind that the older the leaf the farther its distance from the tip of the stem. Since the dis-

TABLE 3

EFFECT OF VARIOUS METHODS OF DEFOLIATION ON FLORAL INITIATION IN BILOXI SOYBEANS SUBJECT TO TWO, FOUR, AND SIX PHOTOPERIODS OF 8 HOURS

LOT NO.	LEAVES LEFT ON PLANTS	TOTAL NODES PER PLANT AFTER VARIOUS PERIODS OF INDUCTION			TOTAL FLOWER-BEARING PLANTS PER LOT OF 10 AFTER VARIOUS PERIODS OF INDUCTION			TOTAL FLOWER BUDS ON MAIN AXIS PER LOT OF 10 AFTER VARIOUS PERIODS OF INDUCTION			LOCATION OF LOWERMOST BUDS CONTAINING FLOWER PRIMORDIA AFTER VARIOUS PERIODS OF INDUCTION		
		2 DAYS	4 DAYS	6 DAYS	2 DAYS	4 DAYS	6 DAYS	2 DAYS	4 DAYS	6 DAYS	2 DAYS	4 DAYS	6 DAYS
1...	All	21.0	21.2	21.7	10	10	10	27	51	69	10.9	10.7	11.0
2...	Both primaries	19.1	18.8	20.1	0	10	10	0	13	31	...	12.0	12.0
3...	One primary	18.4	18.8	19.1	0	10	10	0	12	31	...	11.8	11.8
4...	First compound	20.7	21.2	20.9	0	10	10	0	38	51	...	11.5	11.4
5...	Terminal leaflet of first compound	19.4	19.8	20.3	0	10	10	0	26	45	...	11.3	11.3
6...	Second compound	21.1	21.2	22.0	10	10	10	25	51	69	11.0	10.8	10.6
7...	Terminal leaflet of second compound	19.9	20.6	21.1	10	10	10	22	42	60	11.0	10.8	10.7
8...	Third compound	19.1	19.2	18.6	5	10	10	6	36	45	10.0	9.7	9.9

sections in these experiments were confined to the terminal bud, it is possible that the differences in number of flower primordia formed may have been caused by the increased distance through which the stimulus passed, rather than by a decreased capacity of older leaves to stimulate the production of flower buds. Two experiments were designed to determine the relative importance of these two factors.

The plants used in the first of these studies (experiment 4) were expanding the fourth compound leaf when treatments were started. All plants were decapitated by cutting through internode 5 above

the third compound leaf and were then further defoliated and disbudded in four different ways. In half the plants all buds were removed except the one in the axil of the first compound leaf; in the remaining plants all buds were removed except the one in the axil of the third compound leaf. In half the plants of each of these two lots all leaves except the first compound were removed; in the other half all but the third compound were removed. There were thus four different arrangements of the bud and leaf left on the plant. In one case leaf and bud were both located at node 5; in another both were at node 3; in the third the leaf was at node 5 and the bud at node 3; in the fourth the bud was at node 5 and the leaf at node 3. Twenty plants of each of the four groups were then held at 16-hour photoperiods and twenty more were subjected to 8-hour photoperiods. Ten of these received 4 days of treatment while the remaining ten received 6 days. Since none of the plants receiving 16-hour photoperiods initiated any flower primordia, dissection data from them are not presented.

At the time the experiment was started dissections were made of the buds in the axils of the first and third compound leaves of twenty plants similar to those used for experimental treatment. When the experimental lots were dissected 3 weeks after the beginning of treatment, the total nodes differentiated in the branches developed from these buds were, respectively, 14.8 and 15.2. This represented a gain of 9.7 nodes by the former and 9.5 by the latter. The difference between the two is not significant. Both branches bore flower primordia at an average of 3.3 nodes; thus the branches developed from the buds at nodes 3 and 5 made approximately the same amount of growth and their capacities to form flower primordia were similar.

The data of this experiment (table 4) show that the leaf at node 5 caused the formation of more flower primordia than the leaf at node 3. This difference was present in lots receiving either 4- or 6-day treatments and was uninfluenced by the location of the branch on which the observations were made. In the case of lots 1 and 3, it is evident that the age of the leaf and not its distance from the point of flower bud formation was the cause of the difference in number of flower buds formed. In these lots the more effective leaf was

situated two internodes above the point of attachment of the branch on which flower buds were formed, while the less effective leaf was located at the same level as the point of attachment of this branch.

In the next experiment, designed to differentiate between the effects of age of leaf and relative distance from the growing point, the plants were beginning to expand their fifth compound leaves at the beginning of the experiment and were decapitated at the internode below this leaf. In half the plants all leaves were removed

TABLE 4

EFFECT OF VARIOUS METHODS OF DEFOLIATION AND DISBUDDING ON FLORAL INITIATION IN BILOXI SOYBEANS SUBJECTED TO FOUR AND SIX PHOTOPERIODS OF 8 HOURS

LOT NO.	LOCATION OF LEAF REMAINING ON PLANT (NODE)	LOCATION OF BUD REMAINING ON PLANT (NODE)	TOTAL NODES PER PLANT AFTER VARIOUS PERIODS OF INDUCTION		TOTAL FLOWER-BEARING PLANTS PER LOT OF 10 AFTER VARIOUS PERIODS OF INDUCTION		TOTAL FLOWER BUDS PER LOT ON MAIN AXIS AFTER VARIOUS PERIODS OF INDUCTION	
			4 DAYS	6 DAYS	4 DAYS	6 DAYS	4 DAYS	6 DAYS
1.....	3	3	14.0	15.1	9	10	12	40
2.....	3	5	15.3	15.5	9	10	10	36
3.....	5	3	14.9	15.1	10	10	24	54
4.....	5	5	14.9	15.3	10	10	31	50

except the fourth compound; in the remainder all leaves but the third compound were removed. All buds were taken off the plants except the one produced in the axil of the second compound leaf. This bud was situated one internode below the older of the two leaves studied in the experiment and two internodes below the younger leaf. Treatment with 8-hour photoperiods was continued 4 days in some lots and 6 days in others.

All the plants subjected to 8-hour photoperiods initiated flower primordia, but those with the younger leaves initiated more than those with the older leaves. Since the flower-forming stimulus from the younger leaf traveled approximately twice as far to the point of flower bud formation as did that from the older leaf, it seems evident that the difference in flower-inducing capacities of the two leaves

was related directly to the ages of the leaves and not to their position with respect to the growing points where flower primordia were formed.

In all these experiments the positions as well as the number of flower primordia were recorded. Differences in the location of the first formed primordia were observed among the various lots of certain experiments. These differences also seemed to be correlated with the age of the leaf, and indicated that young leaves were photo-periodically more effective than old ones. In experiment 3 most plants having only the third compound leaf initiated their first flower primordia at node 10. Most of the undefoliated control plants initiated first flower buds at node 11. Other plants having only the primary leaves formed first flower buds at node 12. In one treatment flower bud formation occurred earlier in the development of the plant than it did in the controls; in the other treatment it occurred later.

These differences in location of first flower primordia seem to be caused by the interaction of two factors, the intensity of the flower-forming stimulus produced by the leaves and the rate of development of new structures from which flower primordia could be formed. When only the third compound leaf was used the formation of new nodes was very much curtailed, but the flower-forming stimulus was of sufficient intensity so that the first flowers were formed at node 10, a point on the stem lower than in any of the other treatments. When only the primary leaves remained on the plant new nodes continued to be formed at about the same rate as in the previous case, but the flower-forming stimulus was apparently less intense because the buds at nodes 10 and 11 produced only vegetative structures and the first flower primordia were formed at node 12.

In the controls the first flower primordia were formed at node 11. In this case there was no curtailment of growth because no leaves were removed. Although more flower buds were initiated by these plants than by those having only the third compound leaf, the first flower buds were formed at higher positions on the plant in the former than in the latter. The difference in level was only one node but it was very consistent among all the plants. Differences in the same direction have been found between lots similarly treated in

several other experiments. The reason for this apparently earlier formation of flower primordia on a defoliated than on an undefoliated plant is not yet clear. It seems probable, however, that at the time treatments were started the structures at node 10 had about reached a point in their development beyond which they could no longer respond to flower-forming stimulation. In the case of the controls the primordia at node 10 apparently developed beyond this point before the flower-forming stimulus could reach them and cause formation of flower primordia. In the plants defoliated to a third compound leaf, however, the development of these structures seems to have been retarded or temporarily stopped, permitting the flower-forming stimulus to reach node 10 in time to cause floral initiation when growth at that point was resumed. Whether or not this explanation is correct must be determined by further study.

### Discussion

The data of these experiments suggest that every expanded leaf on a Biloxi soybean plant subjected to photoperiodic stimulation is able to cause the formation of flowers at the growing points. Apparently the various leaves differ considerably in their flower-inducing capacities; but if all are present and subjected to short photoperiods of natural light, the flower-forming stimulus is adequate to result in flower formation in all the new buds formed.

The youngest fully expanded leaf is the most effective one in causing flower bud formation. In experiments 1, 2, and 3 compound leaves 3, 1, and 2 respectively were in this stage of development and caused the initiation of more flower primordia than any other leaf on the plant. Even when they were present alone they induced the formation of as many flower buds as when all the leaves were present. This indicates that they were able to supply the plant with sufficient stimulation to cause floral initiation in all newly formed buds, without the help of the other leaves.

The very young leaves that are just beginning to expand increase very rapidly in their capacity to cause floral initiation—until they are fully expanded. After they pass this stage they apparently supply progressively less flower-forming stimulus.

### Summary

1. Individual leaves on a Biloxi soybean when subjected to photoperiodic stimulation differ in their capacities to cause floral initiation.

2. The most effective leaf on the plant is the one that has most recently attained its full size. Young leaves increase in their capacity to effect floral initiation until they attain full size, after which they gradually decline in effectiveness.

3. The most active leaf, operating alone, is able to cause initiation of as many flower buds per plant as are formed when all leaves function simultaneously.

4. The capacities of different leaves to supply a flower-forming stimulus is correlated with their relative states of maturity and not with their distances from the growing points where flowers are formed. When the flower-forming stimulus from the third compound leaf passes downward through two internodes to the bud in the axil of the first compound leaf, it induces the formation of more flower primordia there than does the stimulus from the first compound leaf which is situated immediately adjacent to the bud.

5. The flower-forming stimulus produced by the leaves of soybeans moves readily both up and down the stem.

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# GROWTH AND DEVELOPMENT OF THE EMBRYO AND FRUIT OF THE PEACH AS AFFECTED BY RING- ING AND DEFOLIATION OF THE BRANCHES<sup>1</sup>

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(WITH FOUR FIGURES)

## Introduction

Peach fruits develop in three stages (13), as shown in figure 1. In stage I the pericarp increases rapidly in size for a definite period following full bloom (about 49 days). The stage is of relatively uniform duration for all varieties, regardless of season of fruit ripening. In stage II, development is retarded for a variable period, depending upon the variety. For a variety which ripens the fruit early, as Greensboro, stage II is of only 5 days' duration; for a mid-season variety, as Elberta, it is 28 days; and for a late-ripening variety, as Chili, it is 42 days. In stage III, known as the final swell, the pericarp increases rapidly in size to fruit ripening. The difference between an early-ripening and a late-ripening variety, therefore, is largely a matter of duration of stage II.

Development of the embryo does not parallel that of the pericarp. During stage I the embryo remains microscopic. At the beginning of stage II the embryo begins a period of rapid enlargement, which continues until it reaches maximum size for the variety. In the case of a very early-ripening variety, stage III is initiated before the embryo has completed the period of rapid development. The embryo fails to fill the integuments, the nucellus and integuments collapse, and a shriveled or abortive seed results. In the case of a late-ripening variety, stage III does not begin until the embryo is nearly the size of the integuments. Such seeds are viable and will germinate following a period of after-ripening.

In an earlier publication (12) it was shown that varieties of sweet cherry (*Prunus avium* L.) and of peach (*P. persica* Batsch.) which ripen the fruit early tend to have abortive embryos. Further, the

<sup>1</sup> Journal article no. 361 of the New York State Agricultural Experiment Station.

earlier the season of fruit ripening the earlier the stage in development at which the embryos are aborted. The question was at that time raised whether early-ripening might induce embryo abortion, or whether abortion might induce early ripening. In a subsequent paper (14) it was shown that destruction of the embryo in stage III hastened ripening of the fruit, thus establishing the point that abortion of the embryo induces earlier ripening, and showing a direct relationship between a developing fruit and a developing embryo within the fruit. A question immediately suggested by these facts is what effect a check in development of the fruit would have in turn upon the development of the embryo within the fruit. The results presented in this paper attempt to answer this question and carry with them certain suggestions as to the part a fruit may play in embryo development.

#### Material and methods

The Elberta and Ward Late varieties of peach were used, the former ripening in late mid-season (128 days after full bloom) and the other late (142 days after full bloom). The studies were conducted during the growing seasons of 1936 and 1937 in the varietal orchards of the New York State Agricultural Experiment Station and in the commercial orchard of Willis Henderson at Dresden, New York. Both were good crop years, providing what would be considered good growing conditions and typical growth for the varieties.

The growth curves of the varieties have been established during several seasons at Geneva, New York. The fact that the blooming period is short (36-48 hours), so that fertilization occurs and fruits begin development at approximately the same time, makes it possible to secure a large number of fruits for sampling which are nearly identical in development at any one time.

Since rapid enlargement and rapid mobilization of storage materials in the embryo do not occur until the fruit has entered stage II, the experimental work was not begun until then. Branches of good vigor,  $\frac{3}{8}$ -1 $\frac{1}{2}$  inches in diameter, and carrying similar numbers of fruits per unit size of branch, were selected. In both seasons the distribution of fruit on the trees was fairly uniform.

Ringings were done with a sharp knife, a ring of bark  $\frac{1}{2}$ -inch wide

being removed, and the exposed wood thoroughly scraped to remove any meristematic tissue which might tend to bridge the gap. Both grafting wax and adhesive grafting tape were used to cover the wounds, both proving equally effective; the latter being more easily applied. All branches in the experiment were inclosed in large cheesecloth bags both to protect the fruit and to catch any which might drop. The fruits per branch varied in number from 11 to 63, with a mean of 27.

Five methods of treatment were used: (1) All current season's wood growth was removed, branch was ringed 2 feet from the end, and all leaves removed from section distal to the ring. (2) Similar to treatment 1 but no leaves removed. (3) No current season's wood growth removed, branch ringed at both 2 and 4 feet from the end, and all leaves removed from the section between the rings. (4) Similar to treatment 3 but no leaves removed. (5) Neither ringed nor defoliated. This combination of treatments made it possible to compare the possible effects from hormones or growth substances manufactured in the growing points of the twigs and the interruption in the movement of materials past the rings. In some instances it was possible to arrange for treatments to be on the same branch, that is, treatments 1, 3, and 5; 1, 4, and 5; 2, 3, and 5; and 2, 4, and 5.

Five sets of experiments were run as follows, the termination of each being determined by the onset of fruit abscission from ringed

## YEAR, PERIOD, AND VARIETY

## DURATION OF TEST

1936 A, Ward Late.....	Beginning of stage II to middle of stage II	
	(July 1)	(July 15)
B, Ward Late.....	Middle of stage II	to end of stage II
	(July 15)	(August 8)
1937 A, Elberta.....	Beginning of stage II to middle of stage II	
	(July 9)	(July 24)
B, Elberta.....	Middle of stage II	to middle of stage III
	(July 24)	.. (August 24)
C, Elberta.....	Middle of stage III	to end of stage III
	(August 24)	(September 18)

and defoliated branches. As will be noted, a new experiment was begun at the time each preceding experiment ended, thus giving a continuous record from the end of stage I through stages II and III

to fruit ripening. The beginning of each experiment with the Elberta variety in 1937 is indicated in figure 1.

Samples of fruit and twigs were collected at both the beginning and end of each period of ringing and defoliation. Fruits, seeds, and embryos were measured and chemical analyses made of twigs, fleshy pericarp, stony pericarp, entire seeds, and embryos. Analyses were also attempted separately of integuments, nucellus, and endosperm, when sufficient material could be secured. Great difficulty was experienced in separating these tissues in quantity and in doing the

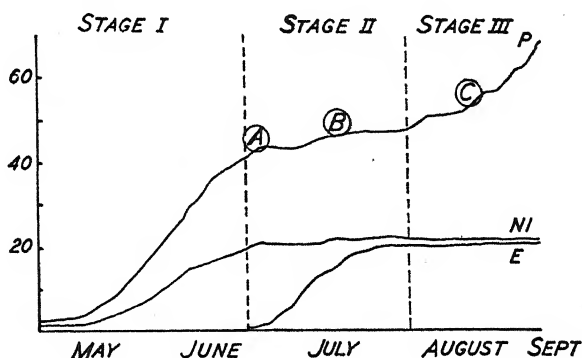


FIG. 1.—Growth of pericarp (*p*), nucellus and integuments (*ni*), and embryo (*e*) of peach from pre-bloom to ripening, showing the three characteristic stages of fruit development and periods at which ringing-and-defoliation (*A*, *B*, *C*) was done.

work with sufficient rapidity to preclude loss of moisture and chemical change.

Determinations were made of total solids, reducing sugars as glucose, sucrose, ether extract (fat), and total nitrogen. In preparing the material for chemical analysis, the seeds were removed from the fruits, and the integuments, remaining nucellus, and endosperm dissected away, leaving the embryos. These were immediately weighed and divided into three duplicate samples for determination of fat, nitrogen, and sugars. The samples for ether extract (fat) were dried at 95° C. for 2 hours and then placed in a vacuum oven at 70° C. until they reached constant weight. The percentage of moisture was determined from these samples. The samples for sugar were placed directly in 80 per cent alcohol.

Fat was determined by the common ether extraction method; sugars by the method involving extraction by 80 per cent alcohol; and sucrose after inversion with invertase. The Munson and Walker method by direct weighing was used for all sugars. The determinations for reducing sugars were calculated as glucose. For nitrogen the Gunning method was used. Calculations were made on the fresh weight, dry weight, and the unit basis.

The results for both seasons and with both varieties are similar. Accordingly complete data are given for the Elberta variety during the season of 1937, as representative, and any variations which occurred with the Ward Late variety and in 1936 are discussed in the text.

## Results

### MORPHOLOGICAL CHANGES

The morphological changes which occurred with the Ward Late variety in 1936 and the Elberta variety in 1937 were similar. There seemed to be no differences in development of fruit or seed, whether on terminal sections of ringed branches or on sections between two rings, whether on ringed or unringed non-defoliated branches, or whether on branches involving several treatments at once (as outlined under Methods). Any hormone or growth substance which may have been manufactured in the leaves or terminal growing points—and which may play a part in fruit and seed development—was either able to pass the rings or was otherwise not affected by the treatments. On the basis of the responses it was thus possible to place the treatments in two groups, (*a*) ringed-and-defoliated and (*b*) check.

The results are shown in table 1. Several points should be emphasized. Ringing and defoliation either checked or greatly retarded the increase in size of the fleshy pericarp, although bringing about earlier coloring and earlier ripening. They retarded the differentiation of the cell walls of the stony pericarp, so that they failed to harden to the same degree as in the check. They brought about earlier differentiation of the integuments, as shown by browning. They failed to have appreciable effect upon the rate of increase and ultimate size of the embryo, as measured in length, up to the time

TABLE 1  
EFFECT OF RINGING AND DEFOLIATION UPON DEVELOPMENT OF  
ELBERTA PEACH EMBRYO (1937)

DATE	LENGTH OF FLESHY PERI- CARP (MM.)	CONDITION OF STONY PERICARP	LENGTH OF NUCELLUS AND INTEG- UMENTS (MM.)	LENGTH OF EM- BRYO (MM.)	DEVELOPMENT (STAGE)
PERIOD A					
July 9 (at time of ring- ing and defoliation).	39.0	Hardening	20.0	2.5	Beginning of II
July 24 On ringed-and-de- foliated branches.	42.0	Less hard than check	20.0	16.0	Middle of II
On check branches..	42.0	Hard	20.0	16.0	Middle of II
PERIOD B					
July 24 (at time of ringing and defolia- tion).....	42.0	Hard	20.0	16.0	Middle of II
August 24 On ringed-and-de- foliated branches.	42.0 (yellow)	Hard	20.0 (integu- ments brown)	19.5	Middle of III
On check branches..	45.0 (green)	Less hard than check	20.0 (integu- ments brown)	19.5	Middle of III
PERIOD C					
August 24 (at time of ringing and defolia- tion).....	45.0	Hard	20.0	19.5	Middle of III
September 18 On ringed-and-de- foliated branches.	47.0 (highly col- ored, lack- ing in fla- vor)	Hard	20.0	19.5	End of III
On check branches..	54.0	Hard	20.0	19.5	End of III

the fruit abscised. But the embryos from ringed-and-defoliated branches seemed less firm than those from check branches, and the weight per unit was less, as shown in table 2 and as discussed later.

TABLE 2

UNIT WEIGHTS OF TISSUES OF FRUIT AND SEED OF ELBERTA PEACH  
ON RINGED-AND-DEFOLIATED BRANCHES;  
FRESH WEIGHT BASIS (MG.)

TREATMENT	PERIOD A		PERIOD B		PERIOD C	
	JULY 7	JULY 24	JULY 24	AUG. 24	AUG. 24	SEPT. 18
FLESHY PERICARP						
Check.....	14,632	18,897	18,897	34,736	34,736	61,306
R and D*....	14,632	15,901	18,897	24,314	34,736	40,589
STONY PERICARP						
Check.....	4748	5690	5690	6108	6108	5340
R and D*....	4748	4847	5690	5208	6108	5396
ENTIRE SEED						
Check.....	638	668	668	690	690	685
R and D*....	638	653	668	646	690	651
EMBRYO						
Check.....	<0.1	349	349	567	567	595
R and D*....	<0.1	340	349	571	567	591

\* Ringed and defoliated.

SHUHART (10) reports somewhat similar results with the pecan, in which defoliation failed to stop the enlargement of the embryo and the filling of the nut.

#### CHANGES IN COMPOSITION

The changes in size and differentiation already discussed are reflected in the changes in weight per unit, in total solids, and in

chemical composition. In table 2 are shown the mean fresh weights per unit, secured by averaging the many hundreds of units taken for chemical analyses. Table 3 gives the mean milligrams per unit of total solids, reducing substances as glucose, sucrose, ether extract, and protein (nitrogen  $\times 6.25$ ), secured by dividing the total amount of a constituent determined in a sample by the number of units contained in it. Percentages on both the fresh weight and dry weight basis have been computed, but since they add nothing significant to the data and since they may be computed from the figures given, they are omitted from this paper.

Although endosperm, nucellus, and integuments were separated from one another in so far as possible and then analyzed, the data are omitted because of the difficulties of sampling as mentioned in the discussion of material and methods. It is possible, however, by subtracting the analyses of the embryo from the analyses of the total seed (table 3), to secure an approximation of the changes which occur in the tissues of endosperm, nucellus, and integument, taken together.

#### CHANGES IN FLESHY PERICARP, STONY PERICARP, AND EMBRYO

FRESH WEIGHT PER UNIT.—The points of particular interest with reference to the fresh weight per unit are (a) the retarded increase in fresh weight of the fleshy pericarp on ringed-and-defoliated branches, (b) the retarded increase and more rapid loss in fresh weight of the stony pericarp, and in sharp contrast (c) the nearly identical increase in fresh weight of the embryo on both ringed-and-defoliated and check branches (table 2 and fig. 2).

TOTAL SOLIDS PER UNIT.—The changes in total solids, or dry weight, shown in table 3 and figure 2 are in many ways the most significant figures in this paper. In the case of the fleshy pericarp, whatever increase in fresh weight did occur on ringed-and-defoliated branches was due entirely to increase in moisture. Actually the fleshy pericarp lost in total solids on ringed-and-defoliated branches as contrasted with the large gain on check branches, at the same time that the total fresh weight per unit was increasing.

In the case of the stony pericarp, the changes are somewhat different from those in the fleshy pericarp and easily may be misinter-

TABLE 3

CHEMICAL ANALYSIS OF TWIGS AND TISSUES OF FRUIT AND SEED OF ELBERTA PEACH ON RINGED-AND-DEFOLIATED BRANCHES IN COMPARISON WITH THE CHECK; UNIT WEIGHT BASIS OF FRESH MATERIAL (MG.)

PERIOD AND DATE	TOTAL SOLIDS		REDUCING SUB- STANCE AS GLUCOSE		SUCROSE		ETHER EXTRACT		PROTEIN (N×6.25)	
	CHECK	R AND D*	CHECK	R AND D	CHECK	R AND D	CHECK	R AND D	CHECK	R AND D
	TWIGS†									
A 7/9.....	5263	5263	139	139	15	15	190	190	206	206
A 7/24.....	5722	5252	157	105	38	23	192	142	200	215
B 7/24.....	5722	5722	157	157	38	38	192	192	200	200
B 8/24.....	5465	4986	142	75	34	8	148	127	175	163
C 8/24.....	5465	5465	142	142	34	34	148	148	175	175
C 9/18.....	6015	5780	132	140	72	15	140	146	206	69
	FLESHY PERICARP									
A 7/9.....	2411	2411	559	559	196	196	70.2	70.2	191	191
A 7/24.....	2834	2083	667	514	306	212	110.0	87.5	303	248
B 7/24.....	2834	2834	667	667	306	306	110.0	110.0	303	303
B 8/24.....	4411	2266	1056	343	848	168	76.4	43.8	349	288
C 8/24.....	4411	4411	1056	1056	848	848	76.4	76.4	349	349
C 9/18.....	7418	3775	2048	958	2520	898	80.3	70.2	526	354
	STONY PERICARP									
A 7/9.....	1051	1051	145.0	145.0	25.6	25.6	23.9	23.9	81.2	81.2
A 7/24.....	2729	1141	51.8	162.0	15.9	13.1	15.4	15.5	127.0	85.3
B 7/24.....	2729	2729	51.8	51.8	15.9	15.9	15.4	15.4	127.0	127.0
B 8/24.....	4446	2720	34.9	18.3	9.8	1.6	15.3	14.1	82.5	39.1
C 8/24.....	4446	4446	34.9	34.9	9.8	9.8	15.3	15.3	82.5	82.5
C 9/18.....	4066	4042	15.0	10.3	5.3	3.2	9.2	9.3	29.3	31.7

\* Ringed and defoliated.

† 10 gm. unit for comparative purposes.

TABLE 3—*Continued*

PERIOD AND DATE	TOTAL SOLIDS		REDUCING SUB- STANCE AS GLUCOSE		SUCROSE		ETHER EXTRACT		PROTEIN (N×6.25)	
	CHECK	R AND D*	CHECK	R AND D	CHECK	R AND D	CHECK	R AND D	CHECK	R AND D
ENTIRE SEED										
A 7/9.....	45.3	45.3	2.8	2.8	0.8	0.8	1.1	1.1	7.9	7.9
A 7/24.....	64.2	65.1	4.5	3.9	2.9	2.6	3.0	3.6	27.4	27.6
B 7/24.....	64.2	64.2	4.5	4.5	2.9	2.9	3.0	3.0	27.4	27.4
B 8/24.....	255.9	177.7	7.5	2.9	9.0	8.9	87.1	38.5	92.3	77.7
C 8/24.....	255.9	255.9	7.5	7.5	9.0	9.0	87.1	87.1	92.3	92.3
C 9/18.....	382.4	325.9	7.0	5.7	13.2	14.7	180.9	125.7	98.3	.....
EMBRYO										
A 7/9.....	< 0.1	< 0.1	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace
A 7/24.....	26.7	27.2	2.8	1.5	1.8	2.1	1.3	1.7	15.1	15.1
B 7/24.....	26.7	26.7	2.8	2.8	1.8	1.8	1.3	1.3	15.1	15.1
B 8/24.....	223.0	152.0	4.6	2.1	7.5	8.5	83.0	34.9	86.5	73.5
C 8/24.....	223.0	223.0	4.6	4.6	7.5	7.5	83.0	83.0	86.5	86.5
C 9/18.....	349.0	294.0	4.4	4.9	12.8	14.2	174.4	120.0	94.0	89.9

puted. On check branches during periods A and B the gain in total solids per unit is actually greater than the gain in total fresh weight per unit. While at first this may seem anomalous, it means merely that the stony pericarp in its hardening processes is losing moisture at the same time that it is gaining in total solids, resulting in a net increase in total weight per unit. For example, while the stony pericarp increased 942 mg. in fresh weight during period A (table 2) it gained 1678 mg. in total solids (table 3). Therefore it must have lost moisture equivalent to the difference between these two figures, or 736 mg. Likewise during period B the gain of 1717 mg. in total solids accompanied by a gain in total fresh weight of 418 mg. means an actual loss of 1299 mg. in moisture during the period. Finally, during period C the stony pericarp lost both in moisture and in total solids,

388 mg. of the former and 380 mg. of the latter for a total loss of 768 mg. per unit fresh weight. The figures thus agree with the morpho-

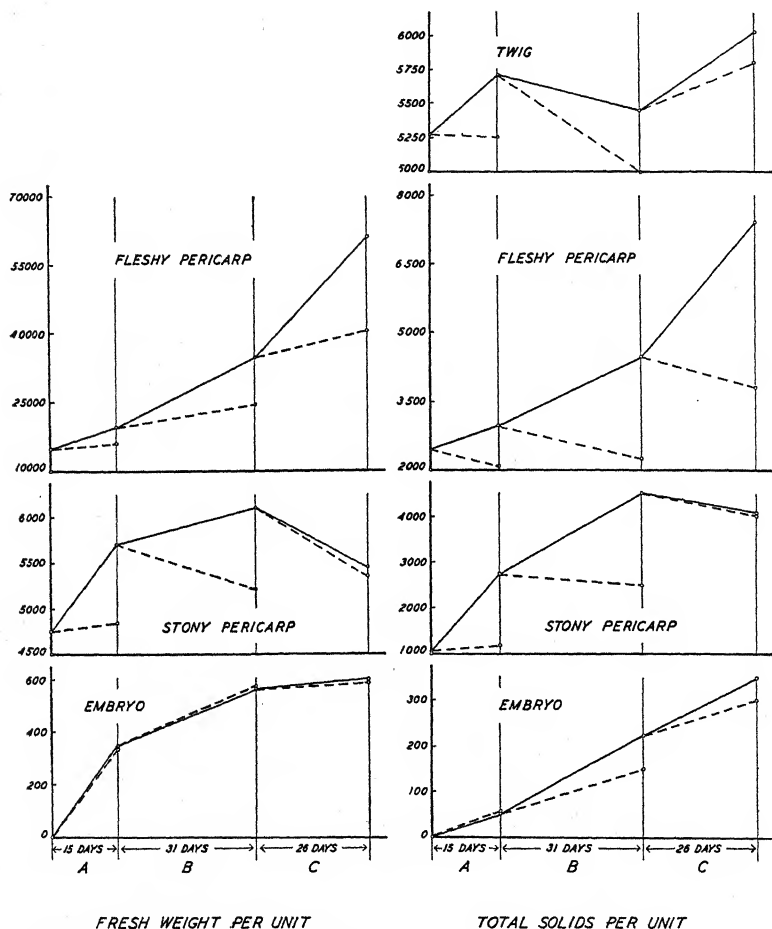


FIG. 2.—Changes per unit (mg.) in fresh weight and in total solids in twigs, fleshy pericarp, stony pericarp, and embryo during three periods of ringing and defoliation (broken line) in comparison with the check (solid line).

logical determinations that the cell walls are greatly thickened and hardened during stage II, and that during stage III there is drying out and increase in brittleness.

The effect on the stony pericarp of ringing and defoliation during

stages II and III was to prevent almost entirely any increase in total solids at a time when such increase was progressing rapidly on check branches. Furthermore, it increased only slightly the loss in total dry weight which occurred in the latter part of stage III on both check and ringed-and-defoliated branches. Here again the figures agree with the morphological findings that the stony pericarp on ringed-and-defoliated branches was checked in differentiation during stage II and did not harden, but was not noticeably affected during the latter part of stage III.

The endosperm, nucellus, and integuments taken together reach the maximum in size and dry weight at the beginning of period A. The moisture content (92.9 per cent) is high, however, the highest of any of the tissues reported here. During subsequent stages on check branches, owing to digestion of the nucellus and later of the endosperm, both the weight per unit and the dry weight steadily declined. Ringing and defoliation increased the loss of solids, particularly during period B, when the embryo was making greatest increase.

The effect of ringing and defoliation upon the embryo is more simply stated. Total solids increased on both ringed-and-defoliated and on check branches, although the accumulation on ringed-and-defoliated branches was not so great as on non-defoliated ones. Whether this was due to a slower rate of accumulation throughout the period, to a retardation toward the end of each period, or to a combination of both, the data give no clue. Since the fruits were left on the tree until they began to absciss, however, one might suspect that the differences were more likely due to a check in accumulation near the end of the period. The figures again agree with the observation that embryos on ringed-and-defoliated branches were more flaccid than those on check branches, even though of equal size and weight.

REDUCING SUBSTANCES AS GLUCOSE PER UNIT FRESH WEIGHT.—Reducing substances (table 3 and fig. 3) increased per unit of fleshy pericarp on check branches during all three periods and decreased on ringed-and-defoliated branches. The greatest loss following ringing and defoliation occurred during period B, when the embryo was making most rapid increase.

In the stony pericarp, reducing substances were found in about the same percentage as in the fleshy pericarp. Instead of increasing in

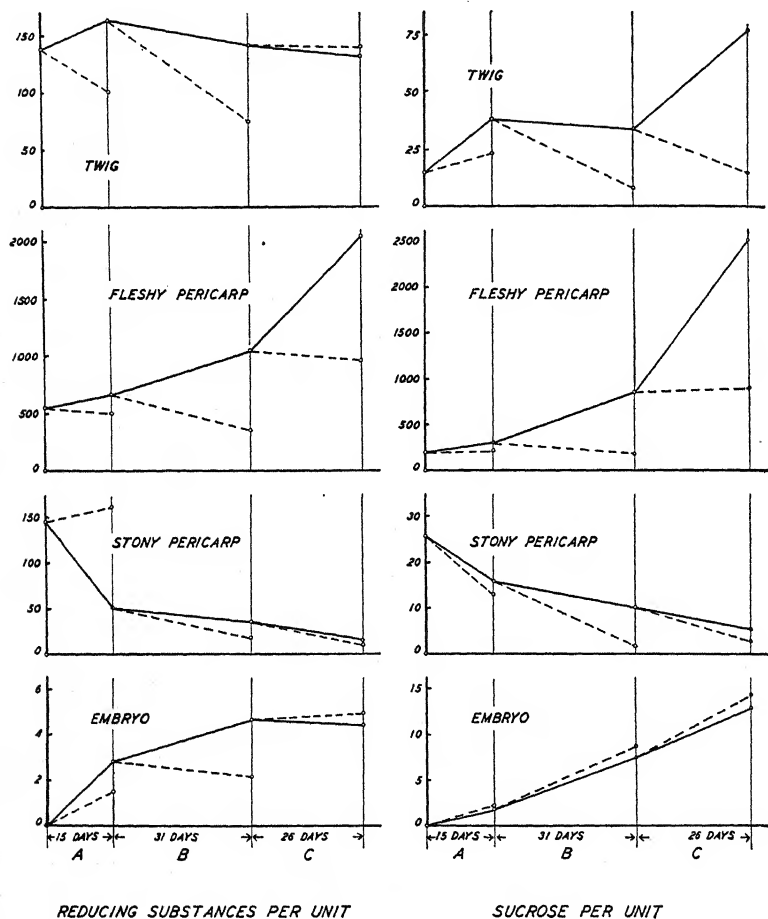


FIG. 3.—Changes per unit (mg.) in reducing substances as glucose and in sucrose in twigs, fleshy pericarp, stony pericarp, and embryo during three periods of ringing and defoliation (broken line) in comparison with the check (solid line).

amount, however, they decreased, both on check and on defoliated branches—except during period A. In that period, both in 1936 and in 1937, the immediate effect of defoliation was to increase the amount of reducing substances in the stony pericarp. No satisfac-

tory explanation of this occurrence or of its significance has been found. The loss of reducing substances during periods B and C on both check and ringed-and-defoliated branches is also of interest.

**SUCROSE.**—The gains and losses of sucrose (table 3 and fig. 3) closely follow those for reducing substances in both fleshy and stony pericarp on check branches. The total amounts of sucrose per unit, however, were slightly less than those for reducing substances in the fleshy pericarp until just before fruit ripening, and much less in the stony pericarp.

Ringling and defoliation failed to retard the accumulation of sucrose in the fleshy pericarp during the first part of stage II. In fact there was a slight increase, suggestive of the increase in reducing substances in the stony pericarp during the same period following like treatment. Subsequently, either the accumulation of sucrose was retarded or else a loss occurred, at a time when there was rapid accumulation in the fleshy pericarp.

In the stony pericarp on check branches sucrose decreased steadily from the end of stage I to fruit ripening, and the decrease was accelerated by ringling and defoliation. In the endosperm, nucellus, and integuments, sucrose was reduced to traces. In the embryo, in contrast, sucrose accumulated steadily during this period on both check and ringed-and-defoliated branches, the accumulation on the latter being even greater than on check branches. It is of interest to note that the effect of ringling and defoliation during the first part of stage II resulted in an increase in sucrose in the embryo as compared with the check, at the same time that it induced an increase in sucrose in the fleshy pericarp and an increase in glucose in the stony pericarp.

**STARCH.**—No starch was found in either the embryo, stony pericarp, or ripe fruit, although small amounts were found in the fleshy pericarp in early stages of fruit development when the fruit was still green. It was possible to secure a value for starch by some quantitative chemical methods for starch determination in tissues other than that of the green fleshy pericarp, but since no starch grains could be detected qualitatively, it is inferred that the value obtained is for some material other than starch. Possibly some of the values reported as starch by other workers may also be for some other material (7, 8).

ETHER EXTRACT.—The amount per unit of ether extract (table 3 and fig. 4) in both fleshy and stony pericarp on check branches was less than the amount of reducing substances. It was also less than the amount of sucrose in the fleshy pericarp and nearly equal to the amount of sucrose in the stony pericarp. There was slight accumulation in the fleshy pericarp during the first part of stage II, followed by decline and leveling off as fruit ripening was approached. The effect of ringing and defoliation was to retard accumulation and to accelerate loss.

In the stony pericarp the amount of ether extract was very low, and during all periods of the experiments there was a steady loss. The losses on check branches were nearly identical with those on ringed-and-defoliated branches. In the embryo, in sharp contrast, ether extract increased steadily on both check and ringed-and-defoliated branches until fruit ripening. At that time ether extract was the greatest single storage constituent of the embryo, 29.31 per cent on fresh weight basis and 49.95 per cent on dry weight basis. The total accumulation was less on defoliated than on check branches. This may have been due to a slower rate of mobilization on defoliated branches than on check branches, or to an earlier cessation of mobilization during this period, or to a combination of both. Since samples were not gathered until the fruit was ready to drop, it would seem that the second suggestion is plausible. The significant point, however, is that the embryo continued to mobilize and to accumulate so much ether extract following defoliation of the branches.

PROTEIN.—The changes in protein (table 3 and fig. 4) in the fleshy and stony pericarp followed in general the trend for sucrose, although the relative quantities of each in the different tissues varied. Thus on check branches there was a slow but steady gain in protein in the fleshy pericarp and an initial gain in the stony pericarp, followed by losses. The effect of defoliation was to reduce the accumulation of protein in the first part of stage II and to increase the losses in the last of stage II and throughout stage III.

In the embryo the trend was similar to that of ether extract, in which there was an increase in protein even on ringed branches. The greatest increase in protein occurred, however, before the last part

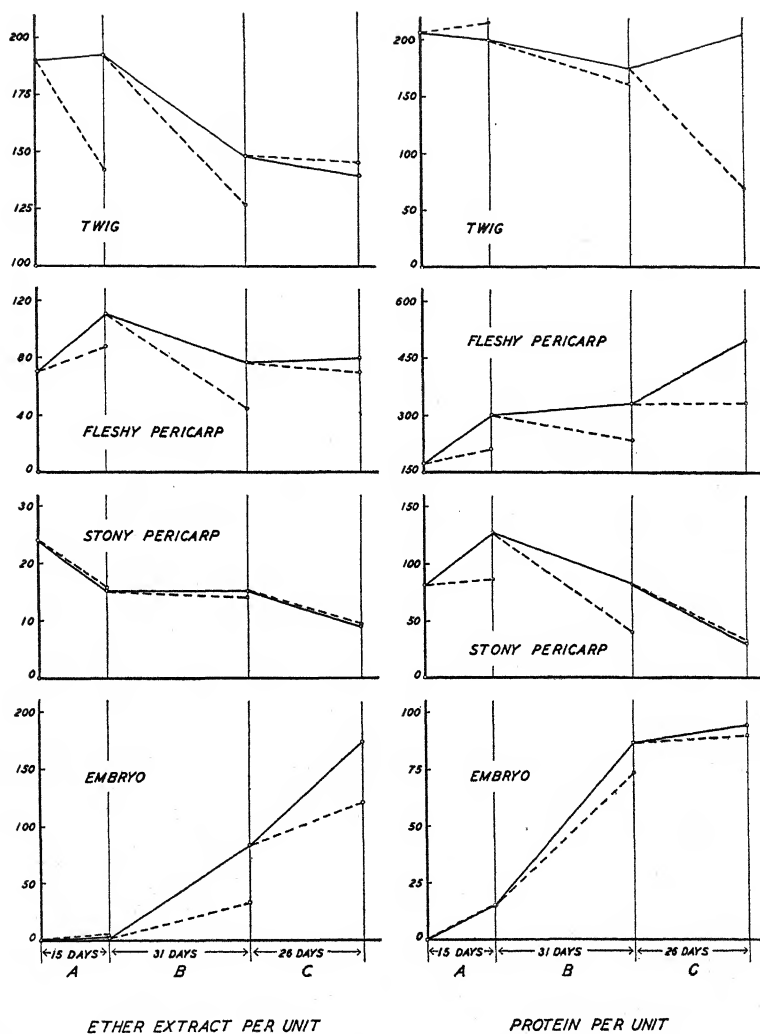


FIG. 4.—Changes per unit (mg.) in ether extract and protein in twigs, fleshy pericarp, stony pericarp, and embryo during three periods of ringing and defoliation (broken line) in comparison with the check (solid line).

of stage III, whereas in the case of ether extract the accumulation continued in about the same amount until the end of stage III. The significant feature is not only that the embryo continued to mobilize protein even after ringing and defoliation, but that the total amounts accumulated on check and on ringed-and-defoliated branches were so similar. Thus during period A the increase on fresh weight basis was from a trace to 4.33 per cent on check branches and to 4.46 on ringed-and-defoliated branches; during period B, 15.26 and 12.88 per cent, respectively; and during period C, 15.80 and 15.20 per cent, respectively.

### Discussion

It has been suggested in the literature that embryos may develop within fruits which have abscised prematurely, indicating that fruit dropping in such instances is not due to failure of the embryo but to the nutritional condition of the tree (2). BRADBURY (1) concluded that embryos of sour cherries may continue development in the embryo sac of an aborting fruit for some time after the ovary has been checked; and TUKEY (15) has shown the potential viability of abortive embryos of apple, pear, peach, cherry, and plum when placed in artificial culture. The data presented in this paper substantiate these suggestions by showing that a check in development of the fruit does not result in immediate check in development of the embryo.

There are implications from the data that even under normal conditions the fruit may play a physiological role and that the materials of the embryo may be derived either entirely or in part from the fruit, as though the fruit served as a reservoir from which materials were moved into the embryo at the time of rapid development. THOR and SMITH (11) have analyzed such a possibility with the pecan. Considering first the seed alone, they conclude "the idea . . . that the kernel of the pecan first fills with carbohydrate material which rapidly changes over to oil during the ripening process is entirely without a quantitative basis." This appears true also for the peach, for the total solids in the entire seed just prior to the period of rapid enlargement of the embryo are only 45.3 mg., whereas the total solids in the embryo alone at maturity are 349 mg.,

to say nothing of their higher energy value compared with that of the materials in the endosperm, nucellus, and integuments.

THOR and SMITH have also shown that it is impossible on a quantitative basis to prove other than "that practically all of the oil content of the pecan kernel is formed from materials brought into the fruit from other parts of the tree at the time of oil formation." VALLEE (16) concluded that the reducing sugars and sucrose which are accumulated in the pericarp of the almond are translocated to the embryo and stored as fat. In studies with the date, LLOYD (6) also considered the possibilities of a nutritive relation between embryo, seed, and carpel. SHUHART (10) has done similarly for the pecan, and LOTT (7, 8) for the peach.

Computations of the data for the peach presented in this paper show that it is theoretically possible for an appreciable quantity of some of the substances which comprise the embryo to be derived from the fruit. Thus on check branches the losses in dry weight from the fruit during development, in fact from the stony pericarp alone, are more than sufficient to equal the increase in dry weight in the embryo. Likewise the losses of reducing substances and sucrose from the stony pericarp are sufficient to equal the increase in the embryo. On the other hand, the losses of ether extract from both the fleshy and stony pericarp together are not sufficient to equal the increase in ether extract in the embryo; yet if the excesses of reducing substances and sucrose above the amount gained by the embryo are calculated as glycerol trioleate, representing ether extract, it is possible to realize a value which makes it theoretically possible for the ether extract gained by the embryo to have been derived from the fruit.

The complication lies in the probable conversion of one form of material into another within the same tissue of the fruit, as suggested by FINCH and VAN HORN (5) and SHUHART (10) for the pecan. Thus the cell walls of the stony pericarp of the peach as here reported become greatly thickened and highly lignified during the period when the greatest loss in dry weight occurs. This loss amounts to 8.5 per cent on dry weight basis and could be accounted for by the dehydration accompanying polymerization of simple materials to more complex ones. If the change was from glucose to a disac-

charide, a loss of 5 per cent could be accounted for; if to cellulose, 10 per cent; while if to lignin, over 10 per cent.

In the case of protein, the loss for the entire fruit does not equal the gain by the embryo, so that the nitrogenous material received by the embryo must come from outside the fruit. On ringed-and-defoliated branches results differ in degree but are similar in nature. Thus the total solids lost by either the fleshy pericarp alone or the stony pericarp alone are sufficient to equal the gain in the embryo. Likewise the reducing substances from both fleshy and stony pericarp are more than sufficient, and the losses in sucrose also. In ether extract the fleshy pericarp alone could supply the material, even without resort to computations involving conversion of reducing substances or sucrose to glycerol trioleate. For protein, as on the check branches, the losses from the fleshy and stony pericarp are not sufficient to equal the gain by the embryo. Only when the losses by the twigs are included are sufficient nitrogenous materials secured to equal the gain by the embryo. Accordingly there can be no question but that the nitrogenous material, at least, comes from outside the fruit.

In the case of a fruit growing under natural environment, it is not possible to say whether materials mobilized in the embryo are derived directly from the endosperm, nucellus, integuments, stony pericarp, or fleshy pericarp and are in turn replaced from outside the fruit, or whether they are derived directly from outside the fruit. The data do not preclude the possibility that the embryo may develop, in part at least, from materials drawn directly from the fleshy pericarp, stony pericarp, endosperm, nucellus, and integuments. It is not necessary, however, to assume that materials which supply the embryo are derived from the fruit, but rather that materials move into both the fruit and the embryo in amounts more than sufficient to supply the embryo, and that when the supply is limited, as by ringed and defoliation, the materials may move from the fruit into the embryo. Such a situation probably occurs on trees low in vigor, or on trees heavily loaded with fruit, resulting in immature, low-quality fruit similar to fruit in these tests on branches which were ringed and defoliated.

MURNEEK (9) has discussed the importance of the developing embryo and has shown in *Cleome spinosa* that developing seeds were responsible for a reduction in growth of the female reproductive organs, thus bringing about sterility. The results of EWART (3, 4) with the pear are also of interest in this connection. He has shown that seedless fruits have a higher sugar content and lower acid content and ripen earlier than seeded fruits of the same variety, and that fruits with high seed content affect the nutritional condition of the tree. From this he has reasoned that trees bearing seedless fruits should be less depleted by fruit production than trees bearing highly seeded fruits. The data presented in this paper carry similar implications.

### Summary

1. The results deal with the development and chemical changes in twigs, fleshy pericarp, stony pericarp, integuments, nucellus, endosperm, and embryo of the Elberta and Ward Late varieties of peaches during the seasons of 1936 and 1937, following ringing and defoliation designed to check development of the fruit and to determine the effect upon development of the embryo. Morphological changes were noted, and determinations were made of total solids, reducing substances as glucose, sucrose, ether extract, and protein (nitrogen  $\times 6.25$ ).

2. The data supplement the results in a previous paper which showed that it is abortion of the embryo which induces early-ripening of the fruit, and not the reverse, by demonstrating that when the supply of materials to the embryo from outside the fruit is limited, as by ringing and defoliation, the embryo continues to increase in size and storage materials continue to be mobilized. At the same time similar materials in the endosperm, nucellus, integuments, stony pericarp, fleshy pericarp, and adjacent twigs either decrease in amount or are retarded in mobilization.

3. The results are discussed with reference to drop fruits, movement of materials between the various tissues, and the physiological role which a fruit may play in the development of the embryo.

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## CYTOLOGICAL INVESTIGATIONS IN DESMODIUM AND LESPEDEZA

J. ORAN YOUNG

(WITH FORTY-THREE FIGURES)

### Introduction

The genera *Desmodium* Desv. and *Lespedeza* Michx. are closely allied taxonomically. They are placed by TAUBERT (12) in the subtribe Desmodiinae, tribe Hedysareae, subfamily Papilionatae of the family Leguminosae. Certain species have been variously assigned to either genus by different workers. At present, however, a fairly constant classification of the species naturally occurring in this country is to be found in the principal floras. Divergences of opinion are chiefly concerned with the specific or subspecific rank of certain forms.

According to TAUBERT, *Desmodium* includes some 160 species, but current investigations indicate that the number is much greater. The genus is widely distributed in both hemispheres, chiefly in North America, southern South America, eastern Asia, subtropical Australia, and South Africa. About forty species are indigenous to the United States but the greatest number of species is found in Mexico. The haploid number of chromosomes has been reported as eleven for four species: *D. perpesium* DC. (6), *D. grandiflorum* (Walt.) DC. (1), *D. canadense* (L.) DC., and *D. tortuosum* (Sw.) DC. (11).

In a recent paper PIERCE (8) gives brief descriptions of the morphology of the somatic chromosomes for twenty-five species and varieties of *Lespedeza*, and summarizes the results of earlier cytological work on the genus. The counts recorded in PIERCE's paper are included in table 2.

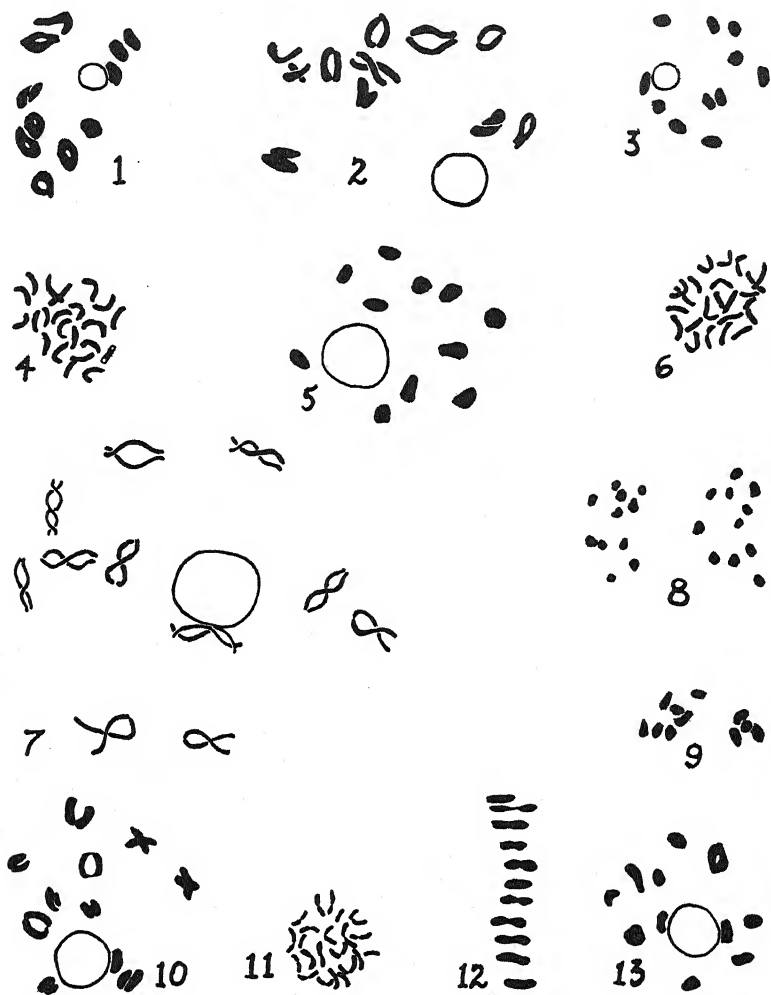
### Material and methods

Most of the material for the present paper was collected along the Atlantic coastal plain and adjacent highland areas, from New Hamp-

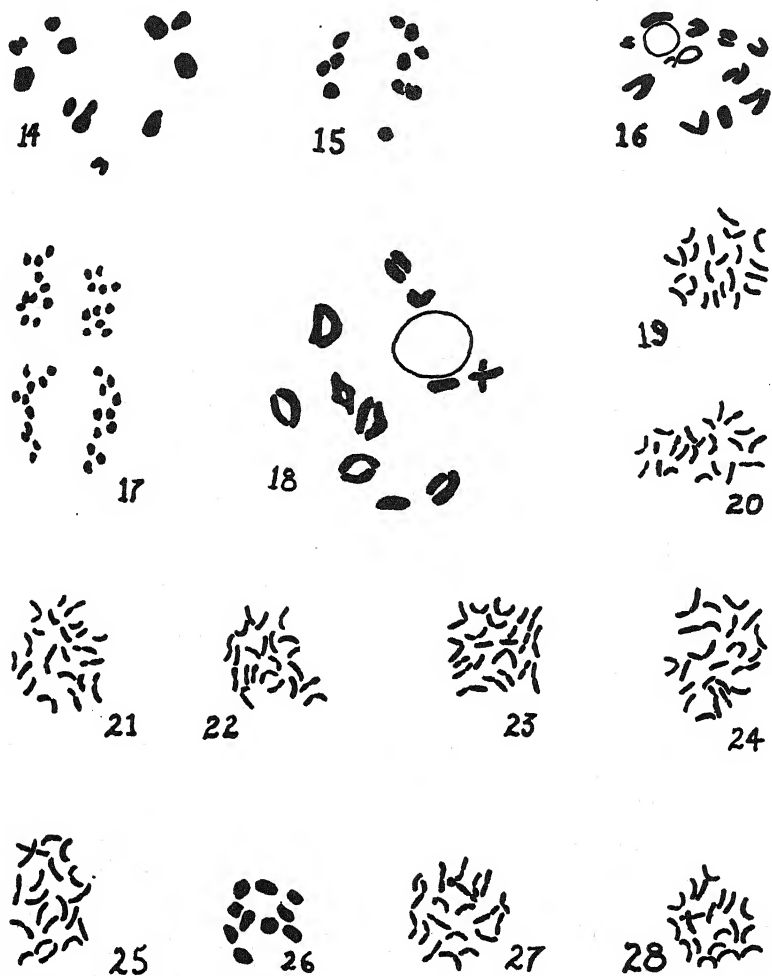
shire to southern peninsular Florida, during the summers of 1938 and 1939. Additional material was obtained north of Lake Seneca in west-central New York state. Seeds of certain species were furnished by the Division of Forage Crops and Diseases, U.S. Department of Agriculture, through the kindness of Mr. Roland McKee. Seeds of *D. triquetrum* DC. were sent by Mr. José I. Otero of the Agricultural Experiment Station, Rio Piedras, Puerto Rico. Mr. Walter V. Brown collected flower buds of three species in New Hampshire. The writer expresses his thanks for these contributions.

Determinations of haploid chromosome numbers were made by the use of iron-aceto-carmin smears. Flower buds were fixed in a mixture of two parts absolute alcohol and one part glacial acetic acid. After 2-5 days the fixative was replaced by 80 per cent alcohol, in which the material was held until the smears were prepared. Difficulty in securing satisfactory differentiation between chromosomes and cytoplasm proved to be a serious handicap. Treatment of the flower buds before smearing with a few drops of hydrogen peroxide in 50 cc. of 80 per cent alcohol or heating the smears at 95° C. for as long as 15 minutes was often of distinct aid in securing better contrast. All figures of meiotic chromosomes are shown at a magnification of about 1100. These were made with the aid of a camera lucida using a 10× ocular and 1.8 mm. apochromatic objective.

For counting the diploid numbers, tips were taken from secondary roots of plants grown in the greenhouse or from primary roots of young seedlings grown on moist blotting paper. On the whole, clearer figures were obtained from secondary roots, but the larger cells of the young primary roots permitted better separation of the chromosomes in some instances. Scarification of the seed coats or exposing the seeds to sub-freezing temperatures usually increased the percentage and rapidity of germination. Figures 4, 6, 19, 25, 28, 32, 36, and 42 are from secondary roots fixed in Navashin's fluid. These figures, at a magnification of approximately 2300×, were obtained by using a 15× ocular and 1.8 mm. apochromatic objective, with the aid of a camera lucida. All other root tip metaphases are from primary roots fixed in La Cour's 2BE. They were drawn with the aid of a camera lucida using a 15× compensating ocular and a 90× apochromatic objective, n.a. 1.40, with a yellow green filter. La Cour's



FIGS. 1-13.—Fig. 1, *D. nudiflorum*, diakinesis. Fig. 2, *D. grandiflorum*, diakinesis. Fig. 3, *D. rotundifolium*, metaphase I. Fig. 4, *D. glabellum*, root tip metaphase. Fig. 5, *D. strictum*, metaphase I. Fig. 6, *D. canescens*, root tip metaphase. Fig. 7, same, diakinesis. Fig. 8, *D. paniculatum*, anaphase I. Fig. 9, *D. dillenii*, metaphase I. Fig. 10, *D. bracteosum*, diakinesis. Fig. 11, *D. rhombifolium*, root tip metaphase. Fig. 12, *D. laevigatum*, metaphase I. Fig. 13, *D. viridiflorum*, late diakinesis. Nucleoli in outline.



FIGS. 14-28.—Fig. 14, *D. canadense*, metaphase I. Fig. 15, *D. rigidum*, metaphase I. Fig. 16, *D. obtusum*, diakinesis. Fig. 17, *D. marilandicum*, anaphase II. Fig. 18, *D. tortuosum*, diakinesis. Fig. 19, *D. incanum*, root tip metaphase. Fig. 20, *D. triquetrum*, root tip metaphase. Fig. 21, *D. latifolium*, root tip metaphase. Fig. 22, *D. discolor*, root tip metaphase. Fig. 23, *D. gyrans*, root tip metaphase. Fig. 24, *D. gyroides*, root tip metaphase. Fig. 25, *L. repens*, root tip metaphase. Fig. 26, same, metaphase I. Fig. 27, *L. procumbens*, root tip metaphase. Fig. 28, *L. violacea*, root tip metaphase. Nucleoli in outline.

2BE fixative produced a slight shrinkage of the chromosomes but gave better definition of the constrictions than could be obtained after Navashin's fluid.

Whenever possible, herbarium specimens have been prepared to accompany the cytological record. In the case of seeds provided by other persons, the writer has accepted their identification but used the nomenclature retained by Index Kewensis.

### Observations

#### DESMODIUM

The species investigated, together with the chromosome numbers and sources of material (or collection numbers if seed was obtained from the U.S. Department of Agriculture), are listed in table 1. The haploid number of chromosomes is eleven in all species studied.

While no attempt has been made to work out the karyotypes of the various species, certain morphological features are apparent. The chromosomes, as seen in somatic metaphases, vary in length from about 1 to 3  $\mu$ . Median constrictions appear to be most frequent, commonly occurring where there is a bend of the chromosomes. Submedian and subterminal constrictions also occur, probably in all species observed. Chromosomes which carry small terminal bodies are interpreted as having subterminal constrictions rather than true satellites.

#### LESPEDEZA

The chromosome counts determined in the present investigation, as well as those of the earlier workers in the genus, are listed in table 2. The morphology of the chromosomes is very similar to that in *Desmodium*. The chromosomes of *L. striata* (fig. 42), however, are decidedly smaller than those of other species observed. In three species, *L. procumbens*, *L. violacea*, and *L. frutescens* (fig. 33), it was possible to observe only nine bivalents in smear preparations of microspore mother cells, although root tip metaphases clearly showed twenty chromosomes. It is possible that the tenth pair of chromosomes was obscured by the nucleolus, which is apparently present until metaphase I or later.

Since the earliest chromosome counts made by the writer for *L. virginica* differed from that recorded by PIERCE (8), many observations were made on meiotic material as well as on root tips

fixed in both Navashin's fluid and La Cour's 2BE. In all, about twenty drawings were made from eight different roots. The clearest

TABLE 1  
CHROMOSOME NUMBERS IN DESMODIUM SPECIES STUDIED

SPECIES	FIGURE	SOURCE OF MATERIAL	N	2N
<i>D. nudiflorum</i> (L.) DC.....	1	Wyalusing, Pa.	II	.....
<i>D. nudiflorum</i> (L.) DC.....		Wilton, Conn.	II	.....
<i>D. grandiflorum</i> (Walt.) DC.....	2	Redding, Conn.	II	.....
<i>D. grandiflorum</i> (Walt.) DC.....		Ossipee, N.H.	II	.....
<i>D. grandiflorum</i> (Walt.) DC.....		Wyalusing, Pa.	II	.....
<i>D. rotundifolium</i> (Michx.) DC.....	3	Junius, N.Y.	II	.....
<i>D. rotundifolium</i> (Michx.) DC.....		Wilton, Conn.	II	.....
<i>D. glabellum</i> (Michx.) DC.....	4	Wilton, Conn.	.....	22
<i>D. strictum</i> (Pursh) DC.....	5	McBee, S.C.	II	.....
<i>D. canescens</i> (L.) DC.....	6	Easton, Conn.	.....	22
<i>D. canescens</i> (L.) DC.....	7	Wake Forest, N.C.	II	.....
<i>D. paniculatum</i> (L.) DC.....	8	Wilton, Conn.	II	.....
<i>D. paniculatum</i> (L.) DC.....		Natural Bridge, Va.	II	.....
<i>D. paniculatum</i> (L.) DC.....		Junius, N.Y.	II	.....
<i>D. dillenii</i> Darl.....	9	Wake Forest, N.C.	II	.....
<i>D. dillenii</i> Darl.....		Wilton, Conn.	II	.....
<i>D. dillenii</i> Darl.....		Swedesboro, N.J.	II	.....
<i>D. bracteosum</i> (Michx.) DC.....	10	Swedesboro, N.J.	II	.....
<i>D. bracteosum</i> (Michx.) DC.....		Redding, Conn.	II	.....
<i>D. rhombifolium</i> (Ell.) DC.....	11	Tavares, Fla.	.....	22
<i>D. laevigatum</i> (Nutt.) DC.....	12	Wake Forest, N.C.	II	.....
<i>D. laevigatum</i> (Nutt.) DC.....		McKenny, Va.	II	.....
<i>D. viridiflorum</i> (L.) Beck.....	13	Swedesboro, N.J.	II	.....
<i>D. viridiflorum</i> (L.) Beck.....		Sumter, S.C.	II	.....
<i>D. canadense</i> (L.) DC.....	14	Nesquehoning, Pa.	II	.....
<i>D. canadense</i> (L.) DC.....		Ossipee, N.H.	II	.....
<i>D. rigidum</i> (Ell.) DC.....	15	Wilton, Conn.	II	.....
<i>D. rigidum</i> (Ell.) DC.....		Estelle Manor, N.J.	II	.....
<i>D. obtusum</i> (Muhl.) DC.....	16	Swedesboro, N.J.	II	.....
<i>D. obtusum</i> (Muhl.) DC.....		Naranja, Fla.	II	.....
<i>D. marilandicum</i> (L.) DC.....	17	Swedesboro, N.J.	II	.....
<i>D. marilandicum</i> (L.) DC.....		Junius, N.Y.	II	.....
<i>D. tortuosum</i> (Sw.) DC.....	18	Jacksonville, Fla.	II	.....
<i>D. tortuosum</i> (Sw.) DC.....		Miami, Fla.	II	.....
<i>D. incanum</i> (Sw.) DC.....	19	Bunnell, Fla.	.....	22
<i>D. triquetrum</i> DC.....	20	.....	.....	22
<i>D. latifolium</i> DC. (= <i>D. lasiocarpum</i> Kuntze).....	21	FPI 103618	.....	22
<i>D. discolor</i> Vog.....	22	FPI 133429	.....	22
<i>D. gyrans</i> DC.....	23	FPI 64038	.....	22
<i>D. gyroides</i> DC.....	24	FPI 77297	.....	22

figures appeared definitely to show twenty chromosomes as the diploid number. It should be noted, however, that it is sometimes difficult to distinguish between one large chromosome with a median constriction and two smaller ones lying end to end.

TABLE 2  
CHROMOSOME NUMBERS IN LESPEDEZA

SPECIES	FIG- URE	SOURCE OF MATERIAL	N	2N	INVESTI- GATOR
Section I. Archilespedeza Taub.					
A. Macrolespedeza Maxim.					
L. bicolor Turcz.			9		Kawakami
L. bicolor Turcz.		81644		22	Pierce
L. cyrtobotrya Miq.			9		Kawakami
L. cyrtobotrya Miq.		FPI 82092		22	Pierce
L. cyrtobotrya var. pedunculata Nakai.		FPI 104066		22	Pierce
B. Eulespedeza Maxim.					
L. repens Bart.		F.C. 21051		20	Pierce
L. repens Bart.	25	Wilton, Conn.		20	Young
L. repens Bart.	26	Estelle Manor, N.J.	10		Young
L. repens Bart.		Roberta, Ga.	10		Young
L. procumbens Michx.		F.C. 85228		20	Pierce
L. procumbens Michx.	27	Wilton, Conn.		20	Young
L. procumbens Michx.		Sylva, N.C.	9*		Young
L. procumbens Michx.		Estelle Manor, N.J.	9*		Young
L. violacea Pers.				20	Pierce
L. violacea Pers.	28	Wilton, Conn.	9*	20	Young
L. nuttallii Darl.	29	Junius, N.Y.	10		Young
L. nuttallii Darl.		Sylva, N.C.	10		Young
L. nuttallii Darl.		Estelle Manor, N.J.	10		Young
L. stuvei Nutt.	30	Estelle Manor, N.J.	10		Young
L. stuvei Nutt.		Sumter, S.C.	10		Young
L. stuvei Nutt.	31	Tifton, Ga.	10		Young
L. stuvei Nutt.		Sag Harbor, L.I.	10		Young
L. stuvei Nutt.				20	Pierce
L. floribunda Bge.		90996		22	Pierce
L. virgata DC.		FPI 90167		22	Pierce
L. frutescens (L.) Britton.	32	Wilton, Conn.		20	Young
L. frutescens (L.) Britton.	33	Redding, Conn.	9*		Young
L. virginica (L.) Britton.	34	Sumter, S.C.	10		Young
L. virginica (L.) Britton.		Estelle Manor, N.J.	10		Young
L. virginica (L.) Britton.		Wilton, Conn.	10		Young
L. virginica (L.) Britton.	35	Central Falls, R.I.		20	Young
L. virginica (L.) Britton.		F.C. 21040		20	Young
L. virginica (L.) Britton.				22	Pierce
L. simulata Mackenzie & Bush.	36	Estelle Manor, N.J.		20	Young
L. capitata Michx.	37	Center Ossipee, N.H.	10		Young
L. capitata Michx.		Wilton, Conn.	10		Young
L. capitata Michx.				20	Pierce
L. capitata var. velutina (Bicknell) Fernald.	38	Estelle Manor, N.J.		20	Young
L. hirta (L.) Hornem.	39	Junius, N.Y.	10		Young
L. hirta (L.) Hornem.		Roberta, Ga.	10		Young
L. hirta (L.) Hornem.		Wilton, Conn.	10		Young
L. hirta (L.) Hornem.		21069		20	Pierce
L. angustifolia (Pursh) Ell.	40	Sumter, S.C.	10		Young
L. sericea Benth.				18	Cooper
L. sericea Benth.			9		Pierce
L. variegata Cambess.		F.C. 21866		18	Cooper
L. variegata Cambess.		F.C. 21866		20	Pierce
L. pilosa Maxim.		F.C. 12086		20	Pierce
L. tomentosa Siebold.				20	Cooper

\* Number observed.

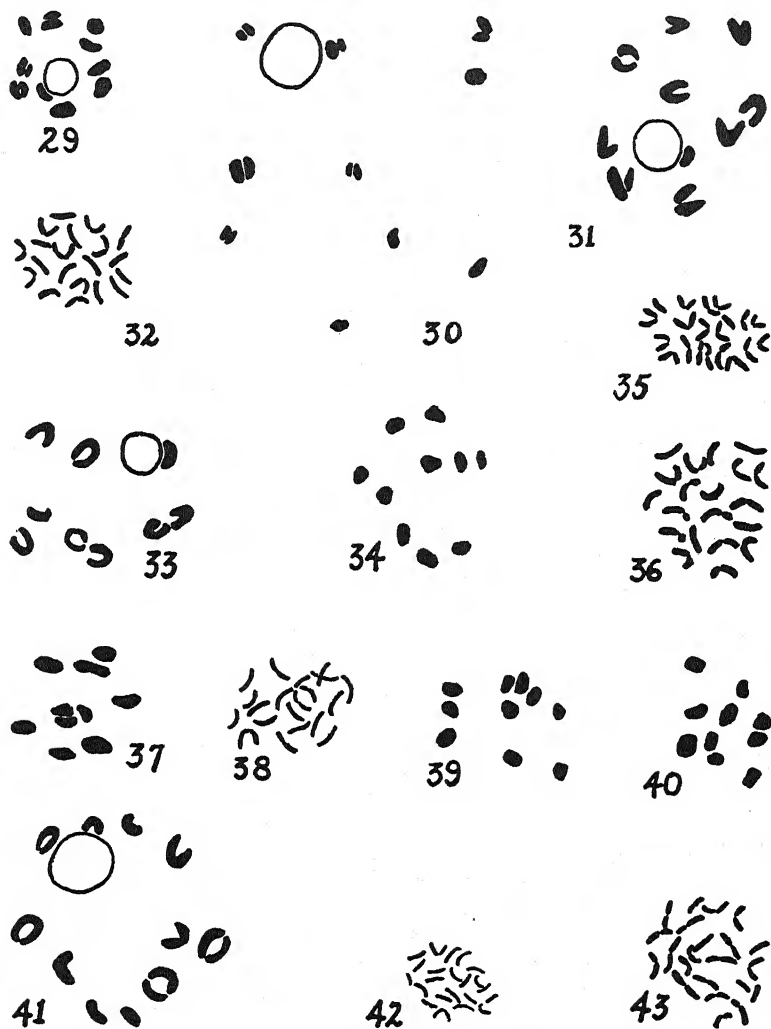
TABLE 2—Continued

SPECIES	FIG- URE	SOURCE OF MATERIAL	N	2N	INVESTIGATOR
Section II. <i>Campylotropis</i> Bge.					
<i>L. macrocarpa</i> Bge.....		F.C. 90998	....	22	Pierce
Section III. <i>Microlespedeza</i> Maxim.					
<i>L. striata</i> var. <i>Kobe</i> .....		F.C. 22242	....	22	Pierce
<i>L. striata</i> (Thunb.) H. & A.....	42	F.C. 22590	....	22	Young
<i>L. stipulacea</i> Maxim.....				20	Cooper
Section undetermined					
<i>L. latissima</i> Nakai.....		F.C. 19283	....	20	Pierce
<i>L. inshanica</i> (Maxim.) Schind.....		FPI 88315	....	20	Pierce
<i>L. maximowiczii</i> Gandog..		82482	....	22	Pierce
<i>L. thunbergii</i> Nakai.....		FPI 25009	....	22	Pierce
<i>L. thunbergii</i> Nakai.....	41	Wilton, Conn.	11	....	Young
<i>L. uekii</i> Nakai.....	43	FPI 108231	....	22	Young
<i>L. japonica</i> var. <i>intermedia</i> Nakai.....		FPI 82094	....	22	Pierce
<i>L. robusta</i> Nakai.....		FPI 108229	....	22	Pierce
<i>L. homoloba</i> Nakai.....			9	....	Kawakami
<i>L. sieboldi</i> Miq. (= <i>L. thunbergii</i> Nakai).....			9	....	Kawakami
<i>L. sieboldi</i> var. <i>albiflora</i> Schneid.....			9	....	Kawakami
<i>L. daurica</i> Schind.....		F.C. 89740	....	36	Cooper
<i>L. daurica</i> Schind.....		F.C. 89740	....	ca. 44	Pierce
<i>L. daurica</i> var. <i>shimadai</i> Masam. and Hosokawa....		FPI 90353	....	ca. 44	Pierce

Specimens referred to *L. nuttallii* from Junius, New York, and Sylva, North Carolina, exhibited the leaf shape and peduncle length of *L. hirta*, together with pubescence characters suggestive of *L. frutescens*. Mature fruiting specimens could not be obtained, but the calyx in both cases resembled that of typical *L. nuttallii* collected in southern New Jersey, where the species is not uncommon. The pollen grains of this material showed an unusual size variation, the smallest having a diameter slightly more than one-half that of the largest.

Specimens referred to *L. stuvei* from Tifton, Georgia, and Sag Harbor, Long Island, New York, are apparently intermediate between this species and *L. nuttallii*. Examination revealed ten bivalents at diakinesis and a notable size variation of the pollen grains. A white form of *L. stuvei* also showed ten bivalents at diakinesis.

Young buds of *L. capitata* var. *velutina* were fixed at Sag Harbor



FIGS. 29-43.—Fig. 29, *L. nuttallii*, diakinesis. Fig. 30, *L. stuvei*, diakinesis. Fig. 31, same (a form resembling *L. nuttallii*), diakinesis. Fig. 32, *L. frutescens*, root tip metaphase. Fig. 33, same, diakinesis, showing only nine bivalents. Fig. 34, *L. virginica*, metaphase I. Fig. 35, same, root tip metaphase. Fig. 36, *L. simulata*, root tip metaphase. Fig. 37, *L. capitata*, metaphase I. Fig. 38, *L. capitata* var. *velutina*, root tip metaphase. Fig. 39, *L. hirta*, metaphase I. Fig. 40, *L. angustifolia*, metaphase I. Fig. 41, *L. thunbergii*, diakinesis. Fig. 42, *L. striata*, root tip metaphase. Fig. 43, *L. uekii*, root tip metaphase. Nucleoli in outline.

and at Smithfield, New Jersey. In material from both sources the anthers had failed to reach full size, evidently withering before anthesis. Pollen grains were small, having an average diameter about two-thirds that of typical pollen grains from *L. capitata*. No meiotic figures could be obtained either in smears or in sectioned material, but the small pollen grains appeared to contain both a vegetative and a generative nucleus. Root tip metaphases showed the diploid number to be twenty-two.

### Discussion

#### DESMODIUM

The chromosome number tabulations of GAISER (2, 3, 4, 5) show that certain families and genera are particularly rich in polyploid species, while others are nearly all, or entirely, diploid. TISCHLER (13) cites the Polygonaceae, Rosaceae, Malvaceae, and Rubiaceae as highly polyploid families, while the Leguminosae and Umbelliferae are families poor in polyploidy. SENN (11) lists forty-two completely diploid genera in the Leguminosae and only ten that are entirely polyploid. In eighteen other genera of the Leguminosae more than half of the species are diploid. Only 23 per cent of the species so far reported in the family are polyploid. When the family is considered in its entirety, it is not surprising to find the genus *Desmodium* completely diploid.

Total absence or very low percentage of polyploidy has been cited for many genera. WOODWORTH (17) and WETZEL (14, 15) list fifteen species and varieties of *Corylus* as diploid, and no polyploidy has been reported; O'MARA (7) cites five species and ten varieties in the genus *Forsythia* as diploid; ROSCOE (9) finds eight diploids in *Wistaria*; SAX and KRIBS (10) describe eleven diploid species in six sections of the genus *Viburnum*; and SENN (11) finds forty-one diploids in *Lathyrus* as against one polyploid.

#### LESPEDeza

Many taxonomists have reported hybrids in the genus and some have been considered of sufficient constancy to receive a place in the floras. Certain intermediate forms that have been examined, however, have not exhibited meiotic irregularity or unusual chromosome numbers. Even though ten species and two varieties of *Lespe-*

*deza*—as well as certain intermediate forms—were found growing in an area about an acre in extent at Estelle Manor, New Jersey, in none of this material were any unusual karyological conditions noted.

A plant collected at Junius, New York, was identified as *L. nuttallii* Darl. WIEGAND and EAMES (16), however, consider forms like *L. nuttallii* found in that region to be hybrids. They state, "Forms occur . . . which agree more or less closely with *L. nuttallii* Darl. and with *L. stuvei* Nutt. These specimens do not constitute distinct categories but present various combinations of the characters of *L. intermedia* Wats. [= *L. frutescens* (L.) Britton] and *L. hirta*. . . . In this region, therefore, the so-called *L. nuttallii* and *L. stuvei* are to be considered hybrids." The specimen collected by the writer at Junius, and a similar one from Sylva, North Carolina, showed ten bivalents and no irregularity other than size variation of the pollen grains. This condition may be related to genetic factors.

If hybridization does occur in *Lespedeza* it is apparently attended by complete compatibility of the parental chromosomes. This hypothesis could be substantiated only by cytological observations on experimentally induced hybrids.

### Summary

1. The haploid number eleven is reported for fifteen species in *Desmodium*. The diploid number twenty-two is reported for nine species.

2. Gametic numbers observed in *Lespedeza* are eleven for one species, ten for seven species, and nine for three species. Where the haploid number nine is reported, the presence of twenty chromosomes in root tip metaphases indicates that the true gametic number is ten. Diploid numbers reported are twenty for six species and one variety, and twenty-two for two species.

3. In both genera the chromosomes vary in length, from approximately 1 to 3  $\mu$ .

4. Pollen grains showed size variation in possibly hybrid species of *Lespedeza*, but no karyological abnormalities were noted.

5. The anthers of *L. capitata* var. *velutina* had withered before anthesis in material from two widely separated localities and only a few unusually small pollen grains were found.

The writer wishes to express his thanks to Professor G. L. CHURCH of Brown University for his generous aid during the course of the problem and to Professor J. M. BEAL for aid in certain critical determinations.

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## SEX EXPRESSION IN WILLOWS

ERNEST C. SMITH

(WITH FOUR FIGURES)

### Introduction

While willows are commonly dioecious, variations from this condition have been observed from early times. LINNAEUS described a monoecious specimen and gave it a specific name, *Salix androgyna*. European literature abounds in mention of what until recently were regarded merely as freaks. PENZIG (18) lists the titles of nearly one hundred papers describing peculiarities of inflorescence in thirty-two species and seven hybrids of this genus. Thirteen papers describe androgynous or gynandrous forms of the European *Salix caprea* and nearly as many report the same conditions in *Salix cinerea*. In the United States reports have been less frequent. The most detailed and complete reports are those of BICKNELL (3, 4) in 1880 and 1881. TWEEDY (26) also described the condition in 1880.

More recently these abnormalities have been carefully studied with the purpose of discovering, if possible, the cause or causes of these variations from the usual dioecious character. HERIBERT-NILSSON (15) in Sweden, RAINIO (19) in Finland, and SCHAFFNER (20, 21, 22, 23, 24) in the United States have approached the subject from this angle.

The writer has met with these abnormal forms in nine species of Rocky Mountain willows, in only one of which has the condition been previously reported.

### Observations

1. *Salix lasiandra caudata* (Nutt.) Sudw. (*S. caudata* Heller var. *bryantiana* Ball.). *Salix* 305 E. C. S.—This shrub is located on the bank of the Cache la Poudre River at La Porte, Colorado, at an elevation of approximately 5000 feet. It is 5 m. in height and entirely normal in all its vegetative characters. It has been observed at different seasons in five consecutive years. Some of the aments contain only staminate flowers, some only pistillate; but more than

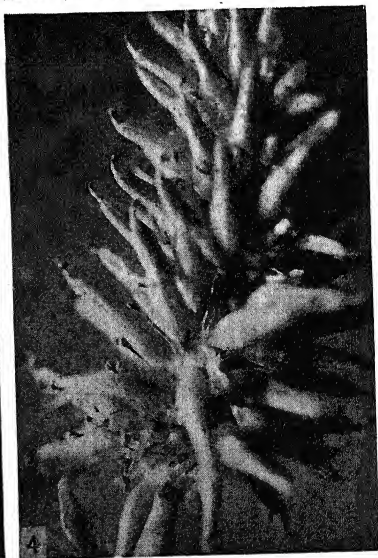
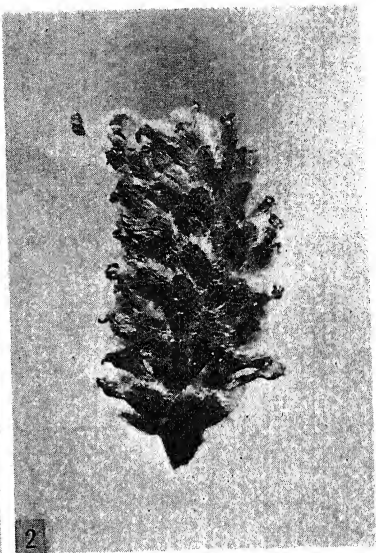
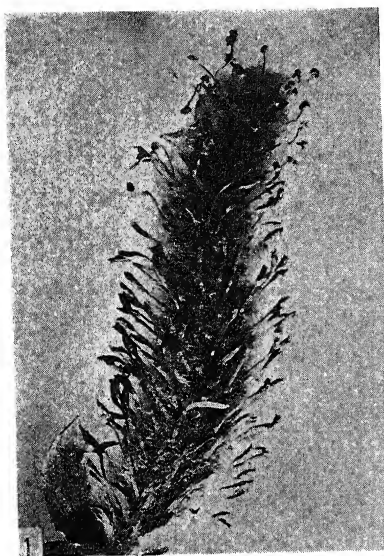
half contain both in varying proportions, the staminate flowers usually occurring at the tip of the ament. A twig taken at random, 3.5 dm. long, bore three aments, the upper two mixed, the lowest wholly staminate. Another twig, 4 dm. long, bore seven aments, three mixed and four pistillate. There are no abnormal flowers.

The same species as no. 305. *Salix* 955 E. C. S. (fig. 3).—This plant is located on the river bank about 2 miles above La Porte. It is a vigorous shrub about 4 m. high. Apparently it is basically staminate, more than half the aments being wholly of that type. As in no. 305, in the mixed aments the staminate flowers occur in the upper portion. The only difference observable is the presence of numerous perfect flowers at the base of the mixed aments (fig. 4). Again there are no abnormal stamens or pistils, only the unusual arrangement of these organs in the catkin. This plant was observed two consecutive years.

2. *Salix lasiandra* Benth., an unnumbered specimen collected by W. W. Jones near Bozeman, Montana, on May 25, 1901.—In the limited amount of material in hand the relative position of the staminate and pistillate flowers in the aments is less fixed than in the preceding individuals. In some aments the pistillate flowers are at the tip and in others the two kinds of flowers are so intermixed that it is only by careful dissection that one discovers there are no perfect flowers present. The stamens and pistils are normal.

3. *Salix scouleriana* Barratt. *Salix* 941 E. C. S.—The shrub is 2 m. tall, at the top of Rist Canyon, 20 miles west of Fort Collins, Colorado, at an elevation of approximately 7500 feet. The aments are pistillate, staminate, and mixed. In the mixed aments the staminate flowers are regularly at the tip. Abnormalities in individual stamens and pistils are sparingly present. Some stamen filaments bear swollen tips which resemble short ovaries and are tipped with stigmas. A few of these shortened ovaries have one lobe transformed into an anther. In a few other cases nonfunctional anthers sprout from between stigmas. Anthers normally glabrous present surfaces with the typical tomentose covering of normal ovaries.

4. *Salix planifolia* var. *nelsoni* Schn. (*Salix nelsoni* Ball).—A single shrub of this species, 2 m. high, was observed in Estes Park, Colorado, at an elevation of 7500 feet. It presented the monoecious character with mixed aments only, the staminate flowers at the tips.



FIGS. 1-4.—Fig. 1, ament of *Salix padophylla* showing stamens changing into pistils. Fig. 2, ament of *S. pseudolapponum* showing incipient change of pistils. Fig. 3, ament of *S. caudata* var. *bryantiana* with staminate tip and prevailing pistillate base. Fig. 4, base of same ament showing perfect flowers (normal stamens and pistils in same flowers).

5. *Salix exigua* var. *stenophylla* (Rydb.) Schn. *Salix* 1008 E. C. S.—Here, instead of single individuals, are nine shrubs showing staminate, pistillate, and mixed aments. They are located near the bank of the Cache la Poudre River, not far from no. 955. Each shrub has from two to four stems averaging 2 m. tall. In all respects except the inflorescence they are indistinguishable from typical specimens, which are also present in large numbers. In the mixed aments the staminate flowers are usually massed at the base, the reverse of the situation in the preceding species, the division between the two kinds of flowers being well marked and abrupt. These plants have been observed for three consecutive years.

6. *Salix petrophila* Rydb. (*S. arctica* var. *petraea* Anders.). *Salix* 998 E. C. S.—Two specimens are growing on a steeply sloping bank above Iceberg Lake in Rocky Mountain National Park at an elevation of about 12,000 feet. Each individual bears two aments. Of these, three have the stamens at the tip, while the fourth has stamens, a few pistils, more stamens, and at the base several pistils. There are no abnormal stamens or pistils.

7. *Salix padophylla* Rydb. (*S. pseudomonticola* var. *padophylla* Ball). *Salix* 940 E. C. S.—A shrub 3.5 m. tall was observed on the bank of Fish Creek, Estes Park, Colorado, at an elevation of 7450 feet. Most of the catkins on this shrub are what HERIBERT-NILSSON (15) calls metamorphosans and RAINIO (19) calls intersexes (fig. 1). Apparently the plant is basically staminate, since it has functional stamens at the tips of a few aments and no functional pistils. The individual flowers exhibit graded stages of sex reversal: Stamens occur with the filaments distinct, but the anthers take on the appearance of shortened and shriveled ovaries. Below these are flowers with the filaments slightly united, then flowers with the filaments completely united. The ovaries formed at the tips of the filaments also display gradations of development, although stigmas are present in nearly all cases.

8. *Salix brachycarpa* Nutt. *Salix* 707 E. C. S.—A single specimen was found growing in moist ground near Poudre Lakes in Rocky Mountain National Park at an elevation of 10,750 feet. This individual also produces abnormal flowers of the type denominated intersexes. There are no normal flowers. Apparently the plant is

basically staminate but in process of transformation into the pistillate condition. Filaments occur, both distinct and united, but instead of anthers they bear at their tips small sacs or cups which are provided with stigmas. Occasionally anthers occur, but in unusual positions, almost sessile or attached to the side or tip of an abnormal ovary which is at the end of a long, filament-like pedicel. Some of the ovaries start near the base of the filaments, in which case they are slender, swelling out only near the tip. While the filaments of this species in normal specimens are glabrous and the ovaries densely tomentose, in these abnormal individuals curious displacements of the pubescence take place—the filaments or portions of them tomentose, the ovaries glabrous or tomentose only on one side. Where anther cells are most completely formed the connective is sometimes tomentose and prolonged some distance beyond the anthers. Under the binoculars the color contrast is striking. Where the union of the filaments is complete and the whole structure bears some resemblance to an ovary, the lower portion is yellow, the tip from purple to dark reddish brown.

9. *Salix pseudolapponum* von Seem. (*S. glaucops* Anders. of Manuals). *Salix* 1255B E. C. S.—A single specimen is growing in moist ground near Poudre Lake in the Rocky Mountain National Park, 7 dm. high. This specimen is of interest because it is the only one seen in which the sex reversal is from the pistillate to the staminate condition. Most of the aments bear normal pistillate flowers. A few bear some staminate flowers at the tip with intersexes where the two types meet. The clearly staminate flowers are not quite normal, the filaments showing the characteristic pubescence of the ovaries. The intersexes show ovaries with the stigmas changing to anthers (fig. 2).

### Discussion

While this last case is the only one observed by the writer showing a change in this direction, BICKNELL (3, 4) and TWEEDY (26) in this country and many investigators in Europe have reported this condition. RAINIO (19) found intersexes so common that he arranged them in five classes of androgynous and five classes of gynandrous forms, basing these upon the degree of change in the organs and on

what he conceived to be the basic condition from which the change started. It will be noticed that in five of the species described by the writer there are no abnormal flowers, the staminate and pistillate or perfect flowers all having normal stamens and pistils. Most reports specify intermediates as occurring at the transition between the two kinds of flowers.

These diverse forms of sex expression are by no means confined to willows. DARWIN (8), observing similar phenomena with a view to their significance for his theory of evolution, stated that "various hermaphrodite plants have become, or are becoming dioecious by many and exceedingly small steps." DAVEY and GIBSON (9) give detailed descriptions of dioecious, monoecious, and intermediate individuals of *Myrica gale*. DARLINGTON (7) lists many species showing these abnormalities, giving data taken from varied sources. SCHAFFNER (20) in his nearly thirty papers in this field includes one specifically dealing with willows.

The current theories of the cause of these abnormalities fall into two classes, one alleging that the causes are external to the plant, or at least have no relation to the segregation and combination of the units of the chromosomes, the other holding that the causes are for the most part internal and dependent upon the genes or upon these in combination with the action of the allosomes.

The most insistent and convincing advocate of the first theory is the late Dr. SCHAFFNER (24). By his experiments with hemp (21), Japanese hop (22), and mulberry (20) he was convinced that dioecious plants, whether staminate or pistillate, carried within themselves the potentialities of expression of the opposite sex (23, 24). He found nineteen out of one hundred individuals of *Salix amygdaloides* showing staminate, pistillate, and intermediate flowers, and applied the conclusions drawn from his experiments with other plants, stating that sex was purely physiological and in no way dependent upon segregation and combination of the units of chromosomes. His emphasis was upon external factors—light, temperature, and soil.

His contention that the potentialities for bisexual expression are present in willows and that external agents may and sometimes do cause sex reversal finds support in the observations of HARRISON (14), who stresses parasitism as an actual cause, stating without

qualification that androgyny in willows is due to castration by Eriophid mites. Distortion of organs due to insect deposition of eggs is a commonly observed fact; that an upset of balance of sex tendencies may be so caused is possible. While the writer has observed a few individual aments of willows in which this is a possible—but not certain—cause of sex reversal, recently direct evidence of modifications due to insect visitations has been evidenced by catkins which have fallen from a staminate specimen of Carolina poplar. Aments falling on May 23, after the leaves were well advanced, exhibited elongated axes and pedicels, forming loose inflorescences in which the disks were much thickened, the filaments very short, but the anthers still distinguishable as such. Aments falling on June 8 showed anthers no longer recognizable as such, partially transformed to ovaries, some of which were provided with stigmas. Sectioning showed the presence of numerous insects, identified at the Entomology Department of the College as mites.

These are not isolated cases. LOEHWING (16) cites numerous reports of similar modifications of sex expression, due not only to insects but also to the indirect action of fungi, which produce chemical substances which have a direct effect.

It must be concluded that willows and many dioecious plants do contain the potentialities for bisexual expression, and that sex reversal and other modifications may be and have been produced by a considerable variety of external agents.

This, however, does not rule out the action of the chromosomes in sex inheritance. SINNOTT and DUNN (25), bringing together data from many sources, state that a cytological basis for sex differences has been found in more than thirty dioecious plants. A statement taking cognizance of both sets of facts is that of ALLEN (1), who states, "There are two obvious facts; first, the potentiality for the production of any character that an organism under any possible condition can manifest must be represented in its hereditary constitution; and second, that this hereditary constitution provides only potentialities, whose expression or non-expression depends on the concurrence of environmental factors."

That some dioecious plants have had sex expression modified by external factors does not prove that these are the only agents which

can and do produce such changes, or that they are the actual agents which produce them under the conditions of natural occurrence. In the case of the willows observed by the writer the action of the external agents previously considered must be ruled out, because the normal and abnormal specimens were growing under identical ecological conditions.

SCHAFFNER takes the dioecious condition for granted and makes no attempt to account for it. If tendencies to expression of bisexual-ity are present in all individuals of the species, how explain the inhibition of one or the other expression in all but a few individuals? If dioecism is a climax character in certain groups of plants, as DARWIN (8) states and BESSEY (2) and many other American botanists believe, then this character is eventually impressed upon the germ plasm. HERIBERT-NILSSON posits an inhibiting factor regularly present in dioecious plants, its occasional absence setting free the tendency to bisexual expression.

As far as willows are concerned, HERIBERT-NILSSON provides conclusive evidence that hybridization is a definite cause of all the abnormalities of sex expression which have been previously described. In this connection RAINIO, who in two seasons of collecting found one hundred and forty individual willows which were abnormal, stated that in the district where he collected, hybrids were as abundant as pure lines. HÅKANSSON (12, 13) made cytological studies of some of HERIBERT-NILSSON's abnormal hybrids and found that they were either triploid or polyploid. These results agree with the findings of BRIDGES (6) and DOBZHANSKY (10) in their investigations of *Drosophila*. They found that triploid intersexes begin as males, develop as such up to a certain point, after which they develop as females. This exactly describes the writer's no. 940 and several of the Swedish scientist's hybrids.

Hybridization is not the only cause of triploidy and polyploidy. DARLINGTON lists seventeen species of plants in which polyploidy has been observed in pure lines. HÅKANSSON reports its occurrence in *Salix aurita*. BLACKBURN and HARRISON (5) proved that at least two hybrid willows adhered to the dioecious condition, and that *Salix caprea*, one of the species used by HERIBERT-NILSSON in his hybridizing experiments, occasionally produced metamorphosans

when cultivated as a pure line. At least five of the specimens cited in this paper show no evidence of hybridization, the vegetative structures being true to specific type and the flowers with normal stamens and pistils. When associated with the perfect flowers one might consider the series as steps in a reversion to an earlier, more primitive type.

The mediating factors in the variations are undoubtedly the hormones. GOLDSCHMIDT (11) states, "Sex factors are substances which cause, take part in, or accelerate a reaction in proportion to the quantity present. The reaction itself is due to the presence of specific hormones of sexual differentiation." It is true that GOLDSCHMIDT worked primarily with insects and we are considering plants; but estrogenic materials have been found by WALKER and JANNEY (27) in the aments of willows. Whether in bisexual or dioecious plants, these hormones act at the points where the stamens or pistils are formed and usually act as completely segregating agents. Granting the presence of tendencies to both staminate and pistillate expression, it is probably the action of the hormones which is most responsive to the influence of external factors and led SCHAFFNER to declare that sex is physiologic rather than genetic.

But back of the action of hormones is always the genetic constitution of the individual, carrying all the potentialities which can find expression under any set of conditions. Apparently in willows there are several critical points for sex differentiation. Normally the form of expression is determined at an early stage and dioecious individuals appear; if the segregation comes later the monoecious condition is in evidence; and if still further delayed perfect flowers are formed. Evidently in the case of intermediates the segregation is delayed so long that the formation of normal stamens and pistils is no longer possible.

### Conclusions

1. Dioeciousness in willows probably expresses a terminal condition or climax in an evolutionary process which is impressed to a high degree on the germ plasm.
2. The potentialities for the expression of both sex tendencies are present in all individuals of the genus, but expression of one of

them is ordinarily in nature inhibited by the genetic constitution. Whether this is due to an inhibiting factor (15), to a realizator factor (17), or to a varying balance between genes and autosomes (6) is not yet clear.

3. Whatever accounts for the dioecious character, some of the variations from that condition are demonstrably due to external agents; others are just as demonstrably due to internal changes. Under the natural conditions in which willows occur, triploidy and polyploidy are common results of hybridization, but occur also in pure lines. In some cases, but not in all, these conditions bring about various abnormalities of sex expression.

4. Even though the underlying causes are in the germ plasm, the action at the point of segregation for stamens and pistils is that of the hormones, and that action may be modified by external factors.

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# DENSITY OF STOMATA AND OIL GLANDS AND INCIDENCE OF WATER SPOT IN THE RIND OF WASHINGTON NAVEL ORANGE<sup>1</sup>

F. M. TURRELL AND L. J. KLOTZ

(WITH NINE FIGURES)

## Introduction

In studies on water spot of the navel orange, FAWCETT, KLOTZ, and HAAS (2) observed that normal fruits with unscratched rinds, when immersed in distilled water for 16 hours, absorbed an average of 1.25 gm. water per fruit. They also noted that: "Water spot of navel oranges affects most severely three locations in the rind: that surrounding the navel, that adjacent to wounds, and the shoulder portion of the stem half" (2). Our observations had indicated that the orange epidermis contained stomata which might afford an entrance for water, and the present investigation was initiated to determine whether stomatal density in certain areas on the fruit surface was related to susceptibility to water spot.

The first symptom of water spot is the appearance of translucent edematous areas in the rind, which later become discolored. Since injection of orange oil into the albedo<sup>2</sup> causes similar discoloration, KLOTZ (4), and FAWCETT, KLOTZ, and HAAS (2) have attributed the discoloration to release of oil from the oil glands. Therefore oil-gland density on the rind surface has also been included in this study.

## Material and methods

Ten orange fruits were picked at random from each of four Washington Navel orange trees located in the plant pathology plots of the University of California Citrus Experiment Station at Riverside. Equatorial and polar diameters were measured with vernier calipers; and on this basis the fruits were separated into two groups, one of

<sup>1</sup> Paper no. 405, University of California Citrus Experiment Station.

<sup>2</sup> The term albedo is derived from the Latin *albus*, white. It has been in use in the citrus industry for some time and refers to the white inner portion of the rind of citrus fruits, differentiating it from the flavedo (yellow), oil-gland-containing outer portion.

large and one of small fruits. The oranges were wiped with cotton saturated with xylene or benzene, and were then similarly wiped with absolute ethanol. Two freehand sections each were cut from the flavedo of the proximal, equatorial, and distal areas. The sections were immersed in a 1:10,000 aqueous solution of ruthenium red for 10-30 minutes, cleared in lacto-phenol, and mounted in the clearing solution.

Five stomatal counts were made at random in each section with a microscope outfitted with a 16.2 mm. objective and a 10 $\times$  ocular. The average number of stomata per microscopic field thus obtained has been reduced to number per square millimeter. Counts were made in 1200 fields.

Oil-gland counts were made on pieces of rind, 8 mm. or more in diameter, cut with a hand razor. Two such surface sections were taken from each of three locations: the proximal, distal, and equatorial areas of the rind of forty fruits picked in a manner similar to that used for the stomatal counts. These surface sections were mounted on glass slides in lacto-phenol clearer and were later transferred to a special counting glass.

The counting glass consisted of two lantern slide covers, 2 $\times$ 2 inches, bound on one edge with cellulose tape to form a hinge. On the outside surface of one of the glasses was cemented a piece of the tape in which a circular hole, 6 mm. in diameter (measured under the microscope), had been cut with a steel spring bow divider.

A section of the rind was placed on the inner surface of this glass directly over the opening in the tape; clearer was added to both surfaces of the rind section; the two glasses were closed together; and the section, thus mounted, was placed in a Leitz VIII S projector equipped with a 250-watt light and an *f*.2.5, 8.5 cm. lens. The image of the circular area of the rind was thrown upon a sheet of paper mounted on a board 263 cm. distant from the lens and showed the oil glands distinctly. Each gland image was numbered on the paper with a pencil, and the final number was recorded. In this manner the oil glands in 240 fields were counted.

Distribution of stomata about oil glands was determined, and an ocular micrometer was used to measure interstomatal and stomatal-oil gland distances. The number of the ring to which a stoma be-

longed with respect to an oil gland as a center and to other neighboring oil glands was also noted. A microscope fitted with an ocular micrometer, a 10×ocular, and a 5.4 mm. objective was used in measuring size of stomata.

### Results

The stomatal apertures of the navel orange fruits averaged 13.0  $\mu$  in length and 5.95  $\mu$  in width. When guard cells were included in the measurements the dimensions averaged 32.4 and 25.5  $\mu$ , respectively. Photomicrographs of stomata are shown in figures 1 and 2.

The results of the stomatal counts are recorded in table 1. They show fewer stomata per square millimeter in the proximal area than in the equatorial or distal areas of all fruits studied, and fewer stomata per square millimeter in large oranges than in small ones.

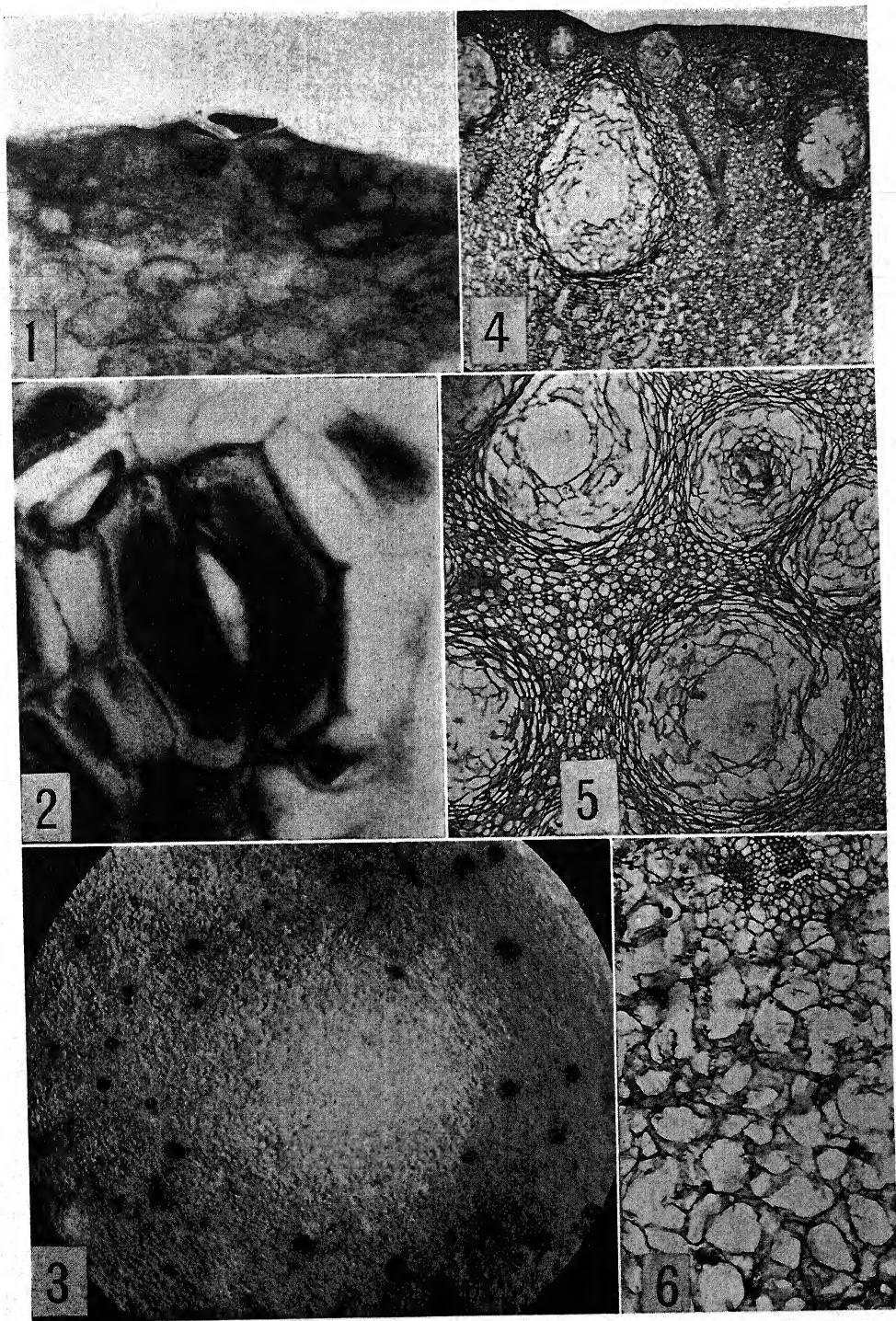
From the mean equatorial diameters and the mean polar diameters of the fruits, the surface area was calculated to be 236.0 cm.<sup>2</sup> for the large fruits and 145.4 cm.<sup>2</sup> for the small fruits. The equation for the surface of a prolate spheroid (3) is

$$S = 2\pi b^2 + \frac{ab}{e} \sin^{-1} e, \text{ where } e = \frac{\sqrt{a^2 - b^2}}{a}.$$

Further calculation showed that large oranges have on the average a greater total number of stomata (284,600) than small oranges (227,700).

From averages of the oil-gland counts, the mean number of oil glands per square millimeter (table 1) was found to be highest in the proximal area, intermediate in the equatorial area, and lowest in the distal area, when all fruits or large fruits were considered. In small fruits, however, the greatest number of oil glands per square millimeter was found in the proximal area, intermediate number in the distal area, and lowest in the equatorial area. The mean of counts in the proximal, equatorial, and distal areas showed higher concentrations of oil glands in small fruits. Sections through oil glands in the flavedo are shown in figures 4 and 5.

REED and HIRANO (5) noted that oil glands on citrus leaves frequently formed a center about which stomata were distributed. This was observed by us to occur in navel orange fruits, although



FIGS. 1-6.—Rind structures of Washington Navel orange fruit. Fig. 1, radial section showing stoma with guard cells and stomatal plug. Fig. 2, surface view showing stoma with guard cells and stomatal aperture. Fig. 3, small portion of rind showing distribution of stomata around oil gland (light central area); stained with ruthenium red. Fig. 4, radial section showing oil glands. Fig. 5, tangential section showing oil glands. Fig. 6, albido below oil glands showing cellular network, large intercellular spaces, hesperidin crystals, and vein.

somewhat less definitely than in leaves. It appeared from a study of large-sized fruits that each oil gland was surrounded by one, two, or more rings of stomata, with five to eight stomata in each ring. This circular distribution is shown in figure 7. The distribution about a single oil gland is shown in figure 3. Although wide deviations in the type of distribution were noted, an average of counts

TABLE 1  
MEAN DENSITY OF STOMATA AND OF OIL GLANDS PER SQUARE MILLIMETER  
IN LARGE AND SMALL FRUITS

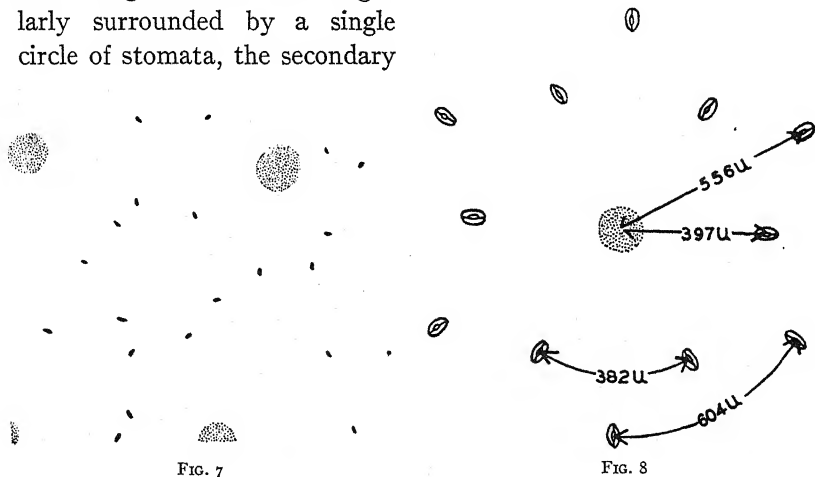
SAMPLE	MEAN EQUATO- RIAL DI- AMETER OF FRUIT (CM.)	AREA			MEAN OF PROXI- MAL, EQUATO- RIAL, AND DISTAL AREAS	PERCENTAGE DIF- FERENCES OF AREAS*	
		PROXI- MAL	EQUATO- RIAL	DISTAL		PROXIMAL AND EQUATO- RIAL	DISTAL AND EQUATO- RIAL
Entire sample.... Large fruits..... Small fruits.....	STOMATA						
	7.49	12.67	14.54	14.38	13.86	-12.8	- 1.1
	8.37	10.79	12.55	12.84	12.06	-14.0	+ 2.3
	6.62	14.55	16.52	15.91	15.66	-11.9	- 3.7
	OIL GLANDS						
	7.13	2.47	2.30	2.22	2.33	+ 7.39	- 3.48
	8.09	2.35	2.21	1.94	2.17	+ 6.34	-12.21
	6.17	2.58	2.39	2.51	2.49	+ 7.95	+ 5.02

\* Plus or minus signs preceding percentages indicate greater or less density of stomata or oil glands in proximal or distal area than in equatorial area.

of stomata and of measurements between oil gland and stomata of the first ring and of the second ring (rings numbered centrifugally from the oil gland), and an average of distances between stomata in any one ring, showed that on the average a much more uniform distribution occurs than can be judged from observation of isolated cases. An idealized representation, based on average measurements, is shown in figure 8.

In twenty-three samples studied, 0-75 per cent (mean 35 per cent) of the stomata forming the first ring about a given oil gland

were included in the first ring of neighboring oil glands, and 0-88 per cent (mean 25 per cent) were included in the second ring of neighboring oil glands. From 27 to 100 per cent (mean 50 per cent) of the stomata in the second ring about a given oil gland might be included in the first rings of neighboring oil glands, but only 0-37 per cent (mean 14 per cent) were included in second rings. This suggests that oil glands are more regularly surrounded by a single circle of stomata, the secondary



FIGS. 7, 8.—Fig. 7 (left), semidiagrammatic camera lucida drawing of distribution of stomata about oil glands, 4.3 $\times$  objective. Fig. 8 (right), idealized representation of distribution of stomata about an oil gland; distances and numbers established as mean of a number of counts.

rings usually being formed by stomata in the first rings of surrounding oil glands.

Examination of both freehand and paraffin sections of untreated flavedo of the Washington Navel orange indicated that numerous stomata were partially or entirely plugged (fig. 1). The plug was generally conical but was frequently observed to be split in the center or unattached to the thick (7.3  $\mu$ ) cuticle. The chemical nature of the plugging material could not be readily ascertained through microchemical tests.

In order to test the effectiveness of the stomatal plugs, whole untreated oranges were immersed in ruthenium red solution overnight. On removal from the solution it was found that many of the stomata,

and frequently the tissues immediately surrounding them, were colored red, but at no time were all the stomata colored. When portions of an orange were repeatedly treated with benzene and finally wiped with alcohol, many more stomata stained with ruthenium red than when the orange was not so pretreated, and practically all in a treated section stained readily after pretreatment. Apparently the stomatal plug is some type of higher hydrocarbon, soluble in benzene, which is effective in preventing water from rapidly penetrating through a substantial proportion of stomata into the pectinaceous, capillary-like albedo (fig. 6).

In this investigation a lesser stomatal density was found in the Washington Navel orange fruits than was observed by REED and HIRANO (5) in the Washington Navel orange leaf (468 per square mm.), and smaller stomatal sizes were noted than those recorded by them (5) for the Valencia orange leaf ( $22.6 \times 17.2 \mu$ ). The mean dimensions of the stomatal aperture of the Washington Navel orange fruits have been found to be smaller than the mean aperture dimensions of stomata observed by ECKERSON (1) in leaves of numerous plants ( $17.7 \times 6.7 \mu$ ), but not so small as the smallest ( $5 \times 2 \mu$ ) found by her in *Cucurbita pepo*.

A moderately high and highly significant negative correlation exists between stomatal density and equatorial diameter of the fruit, as indicated by the correlation coefficient,  $r = -0.597$ . The relation is shown by the regression line (fig. 9) fitted by the method of least squares. Density of stomata may readily be determined from the regression line or from the equation of the line,  $N = -2.78 D + 34.75$ ,  $N$  representing the number of stomata per square millimeter and  $D$ , the equatorial diameter of the orange in centimeters. Such estimations should fall within the standard error of estimate of 4.13 stomata per square mm.

The percentage differences in stomatal and oil-gland density in the proximal area compared with the equatorial area, and in the distal area compared with the equatorial area, are shown in table 2. They indicate that stomatal density is less in the proximal than in the equatorial area, and that it is less in the distal than in the equatorial area, except in large fruits. The difference in the mean stomatal density between the proximal and equatorial areas, when

based on the entire sample, is below the 5 per cent level of significance (table 2) but above the 50 per cent level. Between the means of the distal and equatorial areas the difference in stomatal density is below the 5 per cent level of significance. Based on the mean stomatal density per fruit (average of mean density in proximal, equatorial, and distal areas in each fruit), the difference in the average density in large and small fruits was above the 5 per cent level of significance (table 2).

Oil-gland density is greater in the proximal than in the equatorial area and greater in the distal than in the equatorial area in small fruits, but not in large. Statistical analyses have not been applied to these data; therefore, whether the differences are above the 5 per cent level of significance cannot be stated with surety. The results of the analyses of stomatal density suggest, however, that differences in the mean oil-gland density between different areas on the fruit may also be nonsignificant. Oil-gland density is significantly different in large and small fruits when an average of the mean density in the various areas is used as a basis (table 2). This difference is highly significant since the value of the observed  $t$  is above the 1 per cent level of calculated  $t$ .

Since the incidence of water spot is greatest in the proximal and

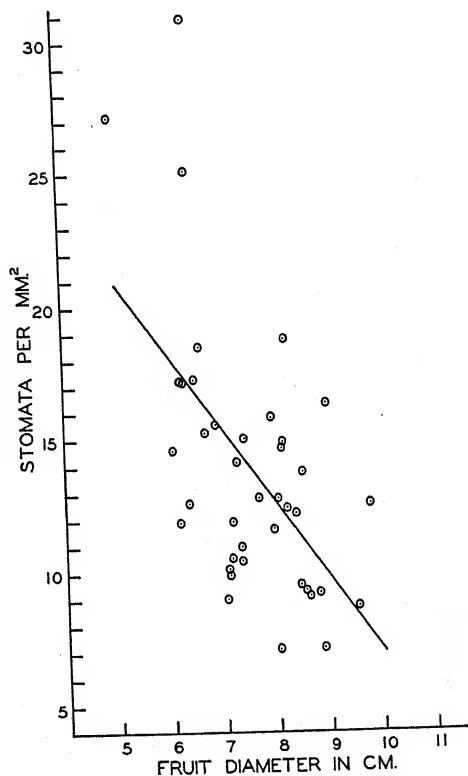


FIG. 9.—Regression line of number of stomata per square millimeter on equatorial diameter of fruit (in cm.).

distal areas (2), it is evident that there is no positive relation between the concentration of stomata and the incidence of this disorder. Oil-gland concentration, however, suggests a positive correlation with the incidence of water spot in the proximal areas of large and small fruits; but the large negative value ( $-12.21$ ) with respect to the distal area of large fruits and the small negative value ( $-3.48$ ) for the distal area of the entire sample indicate no significant correlation with water spot.

Calculation based on data in table 1 showed that small fruits have 29.9 per cent greater stomatal density and 14.24 per cent greater oil-

TABLE 2  
SIGNIFICANCE OF DIFFERENCE OF MEANS OF STOMATAL  
AND OF OIL-GLAND DENSITY

COMPARISON	STANDARD DE- VIATION OF DIFFERENCE	<i>t</i> OBSERVED
Stomatal density (all areas), large fruits and small fruits. ....	1.52	2.36
Stomatal density (entire sample), proximal area and equatorial area.	1.30	1.44
Stomatal density (entire sample), dis- tal area and equatorial area. ....	1.30	0.12
Oil-gland density (all areas), large fruits and small fruits. ....	2.92	3.27

gland density than large fruits. Statistical analyses indicated that these differences were significant. This suggested that the incidence of water spot might be greater in small than in large fruits. When a total of 677 large and small fruits were placed in a rain chamber for 51 hours (91 inches of moisture), however, 47.5 per cent of the small fruits showed water spot in the rind surrounding the navel, as against 50.7 per cent of the large fruits. Similar results were obtained when the navel portions of fifty-two small and forty-one large fruits were immersed in shallow pans of distilled water for 48 hours. As the differences in percentages of injured were not significant, it was concluded that no relation exists between density of stomata or of oil glands and water spot.

### Summary

1. The mean stomatal density for Washington Navel orange fruits was 13.86 per square millimeter. Stomatal density was greater in small oranges than in large. The mean density of oil glands was 2.33 per square millimeter.

2. Oil glands were more densely distributed in small oranges than in large. Neither the density of the stomata nor the density of the oil glands is related to the incidence of water spot.

3. The radial arrangement of stomata in narrow circular zones about oil glands, as reported by previous investigators for leaves of citrus, was also found in the rind of the Washington Navel orange fruits.

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## ORIGIN OF ADVENTITIOUS SHOOTS IN DECAPITATED CRANBERRY SEEDLINGS

HENRY F. BAIN<sup>†</sup>

(WITH FIFTEEN FIGURES)

### Introduction

The hypocotyls of cranberry seedlings (*Vaccinium macrocarpon* Ait.) extend 1-2 cm. above the soil line. If the hypocotyls are severed a few millimeters below the cotyledons soon after emergence of the seedlings, the nodeless stalks almost invariably produce several adventitious buds near their cut ends, one or two of which develop into shoots and carry forward normal growth of the plants (fig. 10). These adventitious shoots originate in the epidermal cell layer.

ROPER (9) first reported the occurrence of adventive hypocotyledonary shoots in 1824, describing them on uninjured plants of *Euphorbia exigua*, *E. heterophylla*, and *E. lathyris*. The phenomenon is now known to occur in many species belonging to widely separated families. RAUH (8) described and illustrated most of the species known to produce adventive buds and shoots on both hypocotyls and roots, and gives an extensive bibliography. He suggests that these plants can be conveniently classified into three broad groups (examples included in the present review produce hypocotyledonary shoots but not necessarily root shoots) as follows: (a) adventive shoots which are obligatory to the development of the plant (*Orobanchaceae*, *Balanophoraceae*, *Rafflesiaceae*, some *Loranthaceae*, *Linaria supina*, *L. bipartita*, *L. heterophylla*, *L. vulgaris*, *L. genistifolia*, *L. striata*, *Euphorbia amygdaloides*, *Linaria alpina*, and *L. reflexa*); (b) adventive shoots which are facultative to the development of the plant, that is, the plant is able to develop to maturity without their formation (annual *Euphorbia* spp., *Antirrhinum majus*, *Linaria spuria*, *L. elatine*, *L. minor*, *Anagallis arvensis*, perennial *Linum* spp., *Alliaria officinalis*, *Isatis tinctoria*, *Anemone silvestris*,

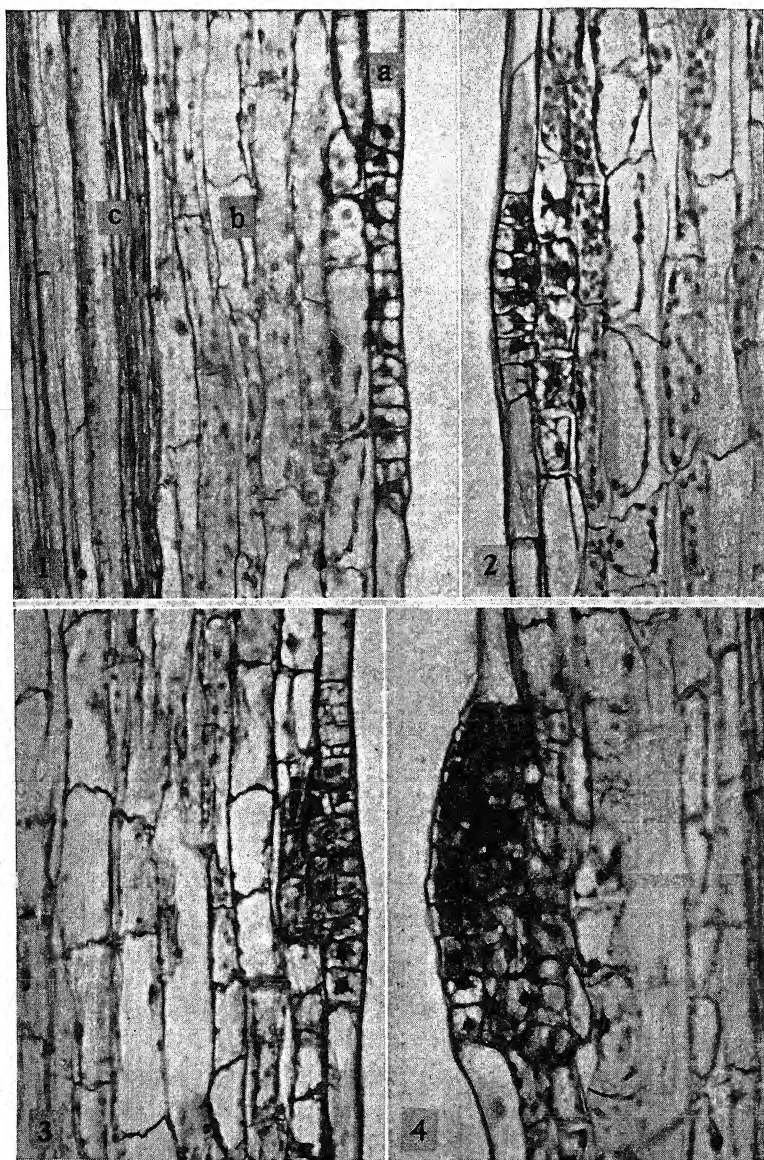
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*Geranium sanguineum*, *Paeonia*, *Pulsatilla*, and numerous trees and shrubs); (c) adventive shoots which are regenerative, that is the shoots develop only when the seedling is injured or excised below the cotyledons (*Euphorbia bubulina*, *Linaria cymbalaria*, and annual *Linum* spp.). The cranberry belongs in the last group, as do tomato (1) and *Annona muricata* (6). The phenomenon of regenerate bud formation on injured hypocotyls may well be more widespread than is realized at the present time.

Hypocotyledonary buds have been found to originate in the epidermis in the majority of the species in which the mode of origin has been investigated. RAUH (8, p. 532) states: "Hypokotylknospen entstehen immer exogen, doch nur an chlorophyllhaltigen, über der Erde befindlichen Hypokotylen, sonst endogen (*Alliaria officinalis*, *Peltaria alliacea*, *Linum flavum*).". He illustrates epidermal bud formation in *Linum usitatissimum*, *L. tenuifolium*, and *Thesium bavarum* (8, fig. 83). CROOKS (4) and BEALS (2) described epidermal bud formation in decapitated flax seedlings (*Linum usitatissimum*). KÜSTER (5) states that buds originate exogenously in decapitated seedlings of *Anagallis coerulea* and *Linaria cymbalaria*. BEIJERINCK (3) described and illustrated exogenous shoot formation in *Linaria vulgaris*, and VAN TIEGHEM (10) confirms exogenous origin in *Linaria* species. PRIESTLEY and SWINGLE (7, pl. 104) illustrated epidermal bud origin in *Linaria macedonica*, but state that according to BEIJERINCK (3) shoot origin in *Convolvulus arvensis* is endogenous.

#### Material and methods

Cranberry seedlings were grown aseptically in test tubes of water agar. After the cotyledons were fully expanded the plants were decapitated 2-3 mm. below the cotyledons with a bent spear-headed needle. Successive lots of stems were then killed in chromo-acetic acid and in Carnoy's fluid at 4-hour intervals, until buds became visible, and afterward at longer intervals until short shoots bearing leaves were developed. The stems were imbedded in paraffin, cut in sections mostly 8  $\mu$  thick, stained in Heidenhain's haematoxylin, and counterstained in Delafield's haematoxylin. Carnoy's fluid yielded the best results on the whole because of its more rapid penetration of the stems.



FIGS. 1-4.—Early stages of adventitious bud formation in cranberry seedlings, as seen in median longitudinal sections of stems. Seed planted January 10; decapitated February 12, 1938. Fig. 1a, epidermal cell divided by cross walls into several short cells, subepidermal cells scarcely affected; b, cortical zone; c, central cylinder. Plant killed February 22. Fig. 2, tangential divisions in epidermal cell, subepidermal cells becoming meristematic. Killed February 22. Fig. 3, subepidermal cells dividing. Killed February 23. Fig. 4, cell activity reaching deeper into cortex. Killed February 23.

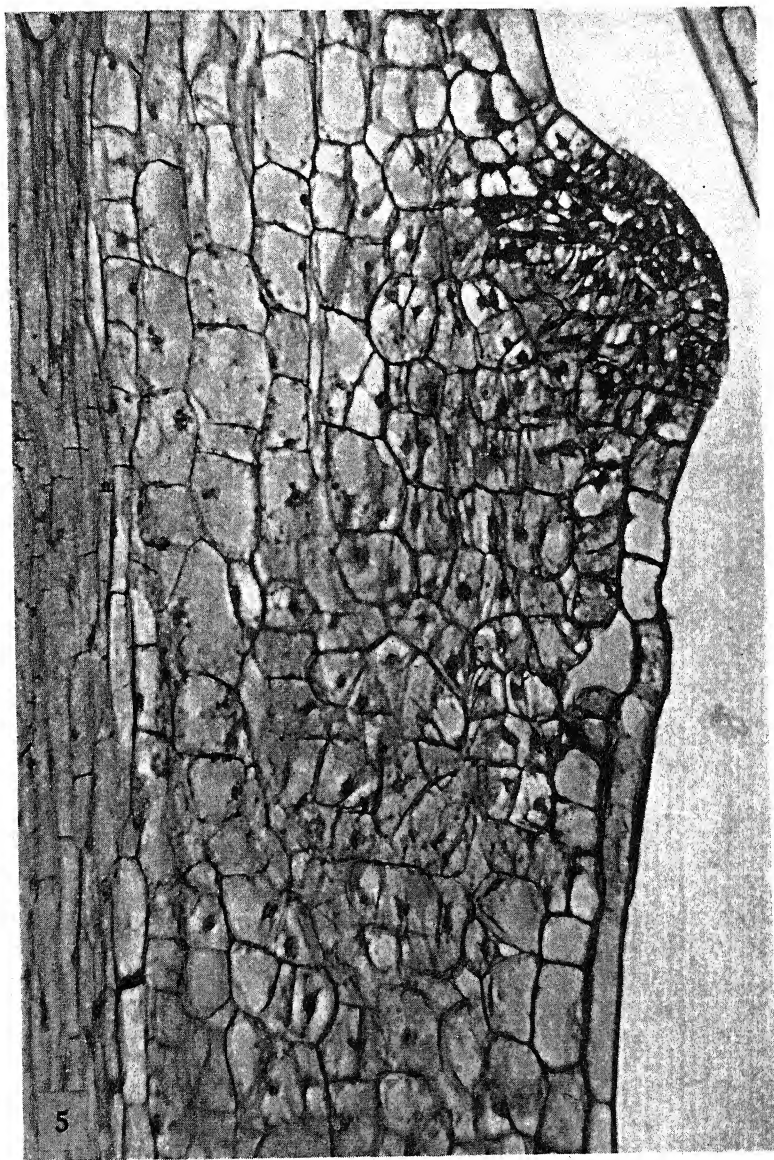
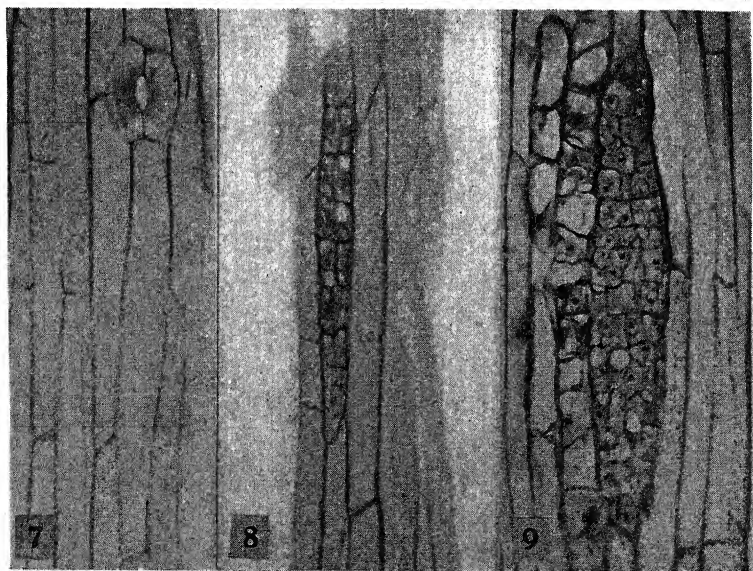
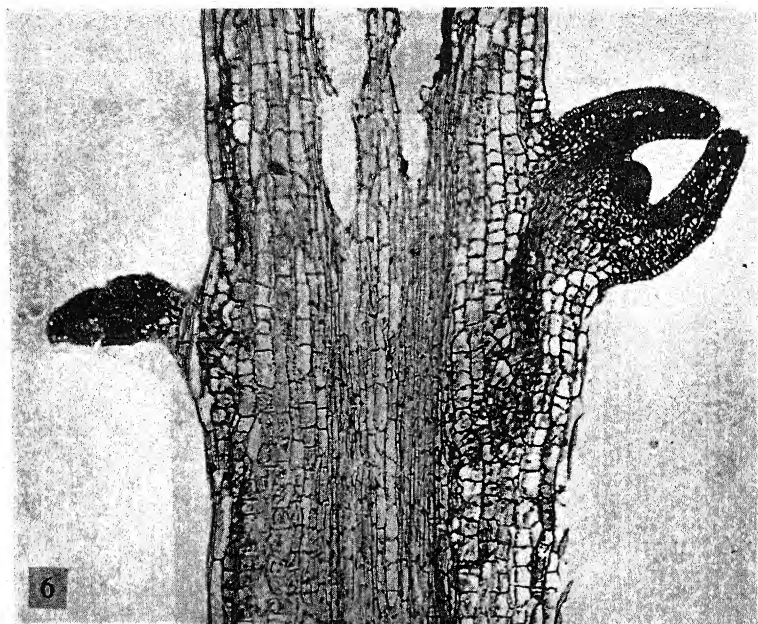
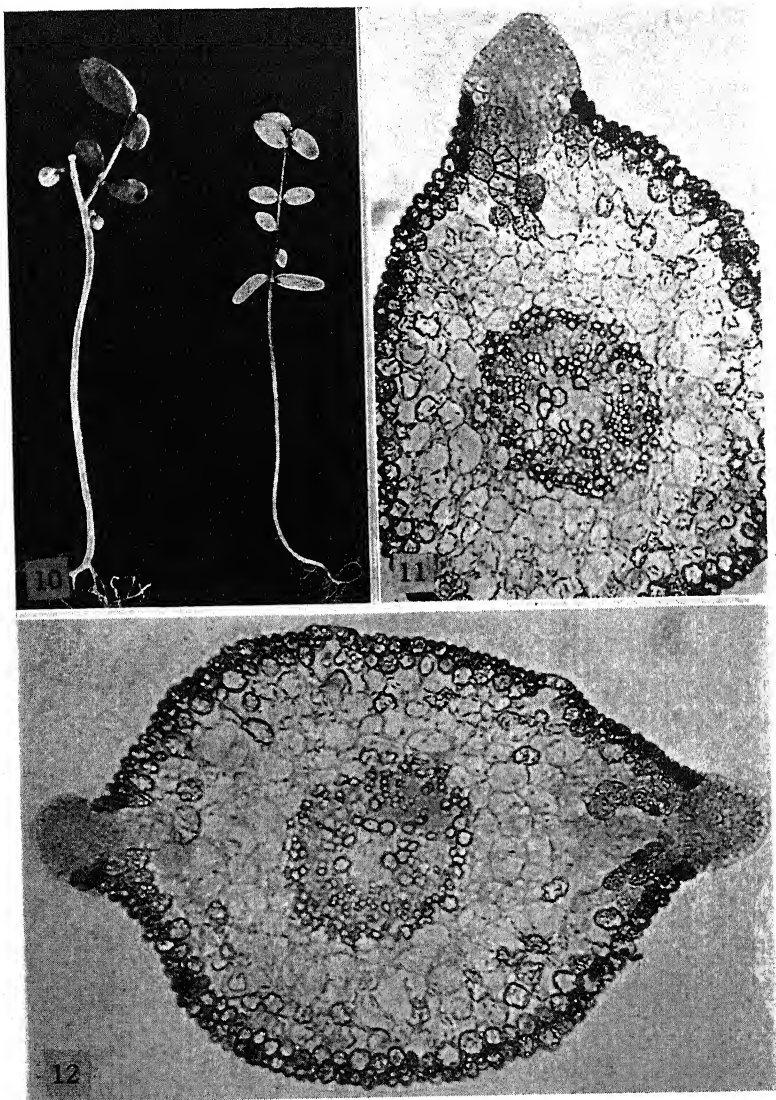


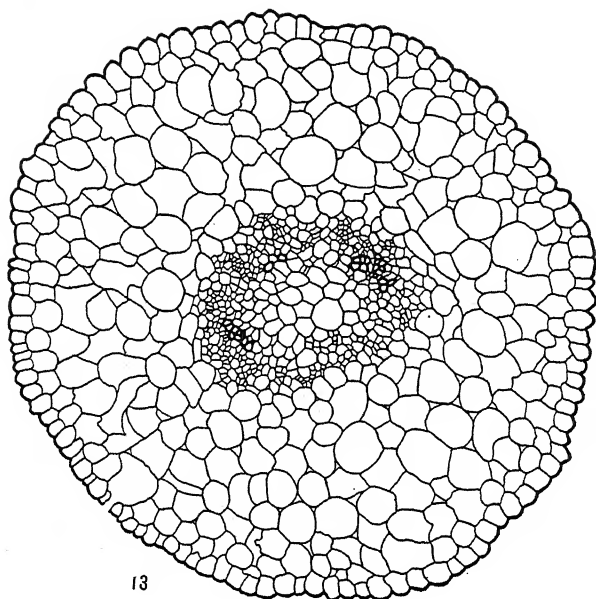
FIG. 5.—Later stage in bud formation, median longitudinal section. Meristematic activity has advanced downward and inward through cortex almost to stem fibro-vascular system. Plant killed 11 days after decapitation.



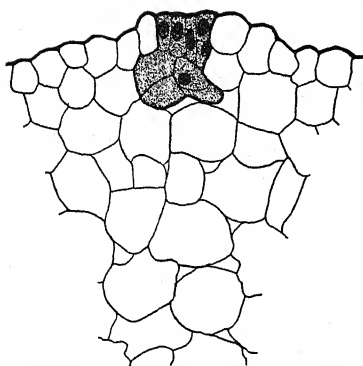
FIGS. 6-9.—Fig. 6, median longitudinal section of stem killed 17 days after decapitation. Fibrovascular system of bud has almost made contact with stem system; second bud on left side of stem is not centrally sectioned. Fig. 7, normal epidermis of hypocotyl as seen in tangential section of stem. Fig. 8, early stage of bud development in tangential section. Within single epidermal cell several cross walls have been laid down, and resulting short cells have undergone one radial division. Plant killed 9 days after decapitation. Fig. 9, later stage showing further divisions in radial plane; adjacent epidermal cells remain unchanged. (Two rows of broad, short cells to left of bud are sub-epidermal cells exposed because the swelling bud had elevated that portion of epidermis



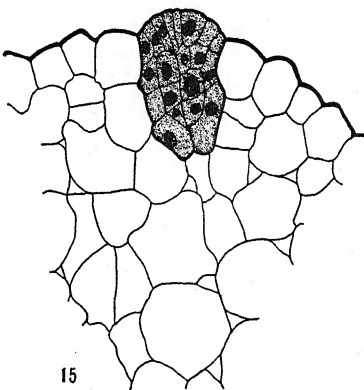
FIGS. 10-12.—Fig. 10, seedlings 4 months after planting in water agar. At left, seedling was decapitated below cotyledons and produced five adventitious buds, three of which produced true leaves, although a single shoot finally became dominant. Normal plant at right. Fig. 11, cross section of adventitious bud in upper third of vertical extension; adjoining epidermal cells pushed aside and cortical cells between bud and central cylinder show no signs of meristematic activity. Plant killed 6 days after decapitation. Fig. 12, cross section  $80\mu$  lower on stem illustrated in fig. 11. Second bud appears at left; bud at right (same bud as in fig. 11) is here cut near bottom of its vertical extension, and at this location meristematic activity, in the form of radial divisions in the cortical cells, can be seen to extend almost to central cylinder.



13



14



15

FIGS. 13-15.—Fig. 13, enlarged drawing from cross section of normal hypocotyl at time plants were decapitated. Fig. 14, early stage of bud development seen in cross section of stem killed 6 days after decapitation. Fig. 15, later stage. Plant killed 6 days after decapitation.

## HYPOCOTYL

At the time of decapitation the hypocotyl apparently consists entirely of primary tissues. The epidermis is composed of narrow, longitudinally elongated cells and contains numerous stomata (fig. 7). Lying next within the epidermis is a cortical zone about six to ten cells deep, consisting of relatively large, thin-walled, highly vacuolate cells containing numerous chloroplasts (figs. 11, 12, 13). The central cylinder has a diameter somewhat greater than one-third the hypocotyl, and contains probably four poorly defined fibrovascular bundles. The cambial ring has not been completed (fig. 13).

## ADVENTITIOUS BUDS

The earliest stage in the development of the buds that could be definitely identified was similar to that shown in figure 1, where a single epidermal cell has been divided by cross-walls into several short cells. Some 2- and 4-celled stages were suspected but in appearance were so similar to the normal irregularly sized epidermal cells that it was uncertain whether the slightly deeper-staining cytoplasmic contents furnished sufficient evidence that active development was beginning. Even the stage reached in figure 1, seen in longitudinal section, could not often be identified in cross sections of the stems.

The meristematic short cells illustrated in figure 1 divide both tangentially and radially (with reference to the stem axis) into extremely small cells (figs. 2, 8). Simultaneously the adjacent cells of the subepidermal layer become active, increasing in size and cytoplasmic content, with the nuclei enlarging (fig. 2). These subepidermal cells soon begin dividing, forming a wedge of meristematic tissue continuous with that initiated in the original single epidermal cell (figs. 3, 4, 14, 15).

Subsequent development takes place outwardly by continued cell division and inwardly toward the central cylinder through meristematic activity of successive cortical cells (figs. 5, 6, 12). The meristematic zone advancing inward to complete connection with the fibrovascular system of the stem follows a downward course through the cortex and eventually makes contact with it some dis-

tance below the developing bud (fig. 6). The available evidence indicates that the outward growth all develops from the tissue originating from a single epidermal cell, adjoining epidermal cells being pushed aside as the bud increases in size (figs. 9, 11, 12).

### Summary

The cranberry belongs to the increasing list of plants for which hypocotyledonary regeneration of growing points has been demonstrated. Adventive shoots in severed cranberry hypocotyls originate in the epidermal cell layer.

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## EFFECT OF NAPHTHALENE ACETIC ACID ON MOBILE AUXIN IN BEAN SEEDLINGS

WILLIAM S. STEWART<sup>1</sup>

(WITH ONE FIGURE)

### Introduction

Application of a 2 per cent lanolin paste of naphthalene acetic acid as a narrow band around the middle of the first internode of 7-day-old kidney bean plants markedly affects their physiology. Some of the treated tissues proliferate rapidly, increasing the stem diameter several times, and there is usually rupturing of the epidermis by many root primordia. In a sufficiently humid atmosphere these primordia develop into roots. The naphthalene acetic acid treatment also causes a mobilization of carbohydrates and nitrogenous materials toward the proliferating tissue. A further effect of the treatment is the inhibition of elongation of the terminal bud (1). It is assumed that these effects are caused by a disturbance in the growth correlation of the plant, and yet no data are available concerning the effect of the treatment on the cell enlargement hormone, auxin, which is known to be related to growth.

WENT and THIMANN (3) restrict the term "auxins," first suggested by KÖGL and HAAGEN-SMIT, to "those substances which bring about the growth reaction which is conveniently measurable by the curvature of *Avena* coleoptiles." As defined, there is no distinction possible between synthetic substances and substances naturally occurring in plants that promote *Avena* coleoptile curvature.

The following investigations were designed to determine the effect of the naphthalene acetic acid treatment on the auxin diffusing from the terminal buds, heart-shaped leaves, and sections of the hypocotyl, and the effect on the auxin diffusing toward these organs from the treated first internode. Auxin collected by diffusion from living organs is spoken of as free-moving or mobile auxin.

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## Experimentation

## MOBILE AUXIN FROM TERMINAL BUDS

Determinations of free-moving or mobile auxin have been made in green plants for leaves, stem tissues, and terminal buds (3). The method was to place the basal cut surfaces of these parts on small agar blocks. The mobile auxin diffuses into the agar and afterwards is quantitatively measured by the *Avena* test. In this investigation somewhat similar technique was used with 6-day-old kidney bean

TABLE 1

MOBILE AUXIN FROM TERMINAL BUDS OF BEAN SEEDLINGS TREATED WITH  
2 PER CENT NAPHTHALENE ACETIC ACID

EXPERIMENT NO.	AUXIN COLLEC- TION (HOURS)	NO. OF BUDS PER PLATE	NO. OF AVENA TEST PLANTS PER DETERMINATION	AUXIN FROM TERMINAL BUDS (AS DEGREES AVERAGE NEGATIVE CURVATURE)	
				UNTREATED	TREATED
52.....	{ First 2	12	24	0	10.8±0.9
	{ Next 5	.....	.....	0	12.6±1.4
	{ Next 21	.....	.....	9.1±1.0	9.0±0.7
61.....	{ First 3½	16	12	5.5±0.9	16.9±1.7
62.....	{ First 3½	24	12	0.0±0	11.7±0.7
	{ Next 23	.....	.....	10.9±1.5	11.0±1.0
63.....	{ First 5	24	12	3.8±1.3	12.2±1.5

plants treated with a ring of 2 per cent lanolin paste of naphthalene acetic acid around the middle of the first internode. (The internodes of 6-day-old, soil-grown bean plants are 2-3 cm. long, the hypocotyl about 10 cm. long.)

The effects of the treatment on the mobile auxin of the terminal bud were first investigated. Two hours after application of the lanolin-naphthalene acetic acid treatment the terminal bud was decapitated with a sharp knife, midway of the second internode which at the time was less than 1 mm. long. The terminal bud was placed for 30 minutes with its cut surface on wet filter paper in a 10-cm. petri dish to remove auxin-destroying enzymes resulting from the cutting of the cells (3). Twelve buds were then placed on a 1.5 per cent agar plate, 6 × 8 × 1.8 mm. After 2-3 hours they were transferred to blank agar plates where they were left for 4-6 hours, before again being transferred to other plates. They remained on these last for

the duration of the experiment. Experiment no. 52, table 1, was performed in duplicate. The first experiments were made with twelve buds per plate. Later the number was increased to sixteen and then to twenty-four. A corresponding number of buds from untreated plants were used as controls (table 1). In figure 1, the mobile auxin is expressed as the amount diffused into agar per hour. The amount of mobile auxin that diffused from the terminal buds of untreated

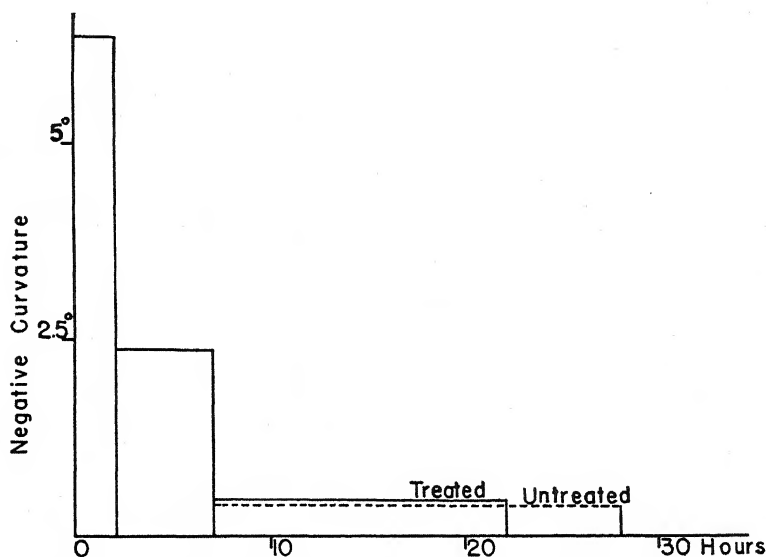


FIG. 1.—Auxin per hour from terminal buds of 6-day-old kidney bean seedlings. Treated by application of 2-mm. wide band of lanolin in 2% naphthalene acetic acid around middle of first internode 2 hours prior to removing buds from plant.

plants was so small that it was detectable only if it was collected for a period of 15 hours. A larger amount of mobile auxin diffused from the terminal buds of treated plants. It is probable that this auxin may be in part naphthalene acetic acid.

#### MOBILE AUXIN FROM LEAVES

Mobile auxin from the heart-shaped leaves of 6-day-old bean seedlings was determined by removing them from the plant by a clean cut at the base of the petiole after the 2-hour treatment with naphthalene acetic acid encircling the first internode (the average area per leaf was 11 sq. cm.). The entire leaves were then immersed

for 30 minutes in tap water at the same temperature as the greenhouse where the plants had been growing ( $24^{\circ}\text{C}$ ). They were then put between two pieces of water-soaked cellucotton and placed on a stand 1 inch high. The stand was made from  $\frac{1}{4}$ -inch mesh wire screen. The petioles projected downward about  $\frac{1}{2}$  inch through the meshes of the screen. To keep the blades flattened, a piece of glass was put over the top layer of the cellucotton. A 1.5 per cent agar block  $2 \times 2 \times 1.8$  mm. was placed on the cut surface of each petiole. The stand was placed in a glass chamber lined with wet filter paper

TABLE 2

MOBILE AUXIN FROM HEART-SHAPED LEAVES OF BEAN SEEDLINGS  
TREATED WITH 2% NAPHTHALENE ACETIC ACID

EXPERIMENT NO.	AUXIN COL- LECTION (MINUTES)	NO. OF LEAVES PER DETERMI- NATION	NO. OF AVENA PLANTS PER DE- TERMINATION	AUXIN (AS DEGREES AVERAGE NEGATIVE CURVATURE WITH STANDARD ERROR)	
				UNTREATED	TREATED
68.....	105	30	30	$6.6 \pm 0.5$	$10.9 \pm 0.9$
70.....	135	21	21	$9.1 \pm 1.0$	$11.3 \pm 1.0$
109.....	180	50	50	$12.2 \pm 0.5$	$11.6 \pm 0.5$
110.....	105	40	40	$10.1 \pm 0.5$	$8.1 \pm 0.4$

and kept in a dark control room at  $24.5^{\circ}\text{C}$ . By this technique mobile auxin from the petiole was collected in the agar block and determined quantitatively by the *Avena* test (table 2). The amount of auxin which diffused from leaves of treated plants was not significantly different from the amount diffusing from leaves of untreated plants.

#### MOBILE AUXIN FROM HYPOCOTYL

The amount of auxin which diffused from sections of the hypocotyl from treated plants was compared with the amount from untreated plants. Sections 1 cm. long were cut from the middle and base of the hypocotyl. They were placed upright on moist filter paper as described for the terminal buds and then transferred to 1.5 per cent agar plates  $6 \times 8 \times 1.8$  mm. in size. Another agar plate of the same size was placed on the upper surfaces. Five sections per plate were used. In experiment 72B after  $2\frac{1}{2}$  hours, and in experiment 74 after  $4\frac{1}{2}$  hours, the plates were cut into twelve blocks of equal size

and analyzed for auxin. There was more auxin from the mid-hypocotyl sections of treated plants than from untreated ones (table 3), but no significant difference for the basal sections.

Whether the increased amount of auxin was owing to increased production of a naturally occurring mobile auxin, perhaps from some precursor, or whether it was auxin coming from the externally applied naphthalene acetic acid was a matter of question.

TABLE 3  
MOBILE AUXIN FROM PIECES OF HYPOCOTYL OF BEAN SEEDLINGS  
TREATED 2 HOURS WITH 2 PER CENT NAPHTHA-  
LENE ACETIC ACID

EXPERIMENT NO.	AUXIN COLLECTION FROM HYPOCOTYL	AUXIN (DEGREES AVERAGE NEGA- TIVE CURVATURE OF TWELVE AVENA TEST PLANTS)	
		UNTREATED	TREATED
74.....	Middle section Upper surface	$0 \pm 0$	$7.6 \pm 0.8$
74.....		$2.3 \pm 1.0$	$7.1 \pm 1.6$
72B.....		$3.6 \pm 1.0$	$12.1 \pm 1.1$
74.....	Middle section Lower surface	$6.5 \pm 1.4$	$9.3 \pm 0.5$
74.....		$1.8 \pm 0.9$	$6.5 \pm 1.6$
72B.....		$5.4 \pm 0.8$	$17.3 \pm 0.8$
74.....	Basal section Upper surface	$0 \pm 0$	$0 \pm 0$
74.....		.....	$0 \pm 0$
72B.....		$4.7 \pm 1.1$	$1.5 \pm 0.8$
74.....	Basal section Lower surface	$1.6 \pm 0.8$	$6.8 \pm 0.7$
74.....		$5.4 \pm 0.7$	$7.1 \pm 0.7$
72B.....		$0 \pm 0$	$3.6 \pm 1.2$

MOBILE AUXIN DIFFUSING TOWARD TERMINAL  
BUD, LEAVES, AND HYPOCOTYL

Since the naphthalene acetic acid treatment caused an increase in the mobile auxin diffusing out of the terminal buds and hypocotyl sections removed from the plant, the question was raised as to whether or not this auxin came from the first internode, the site of application of the naphthalene acetic acid. Accordingly the mobile auxin diffusing away from the first internode and toward these organs was measured.

Six-day-old kidney bean plants were grown in soil as in the first experiments. They were then selected for uniformity of size and

treated at the middle of the first internode with 2 per cent lanolin paste of naphthalene acetic acid as before. Immediately after treatment a 3 per cent agar block ( $2 \times 2 \times 1.8$  mm.) was put at one of the following places on the plant: (a) on the stump of the second internode after decapitation of the terminal bud; (b) on the stump (about 1 mm. long) of the petiole after removal of one of the heart-shaped leaves; (c) on the cut end of the midrib of the leaf blade about 2 mm. beyond the junction of the petiole and blade;<sup>2</sup> (d) on the remaining stump of one of the cotyledons after its removal; and (e) on the lower half of the hypocotyl in a small incision about 1 mm. deep. The incisions into the leaf and hypocotyl were made under water to prevent air from entering the vessels. Twelve plants were used for each location of the agar blocks. The experiment was repeated for the petiole stump, leaf blade, and hypocotyl. In all cases as soon as the agar block was in position the whole plant was covered with a glass jar lined with moist paper. During days of bright sunlight the plants were shaded. The agar blocks were allowed to stay on the plants for 2 hours, the same length of time allowed for treatment in the first series of experiments. They were then tested for auxin. Generally agar blocks from comparable positions on treated and untreated plants had dried out to the same extent, so that direct comparisons of the auxin could be made. The size of the block usually did not decrease more than half. The greatest drying occurred with agar blocks applied to the hypocotyl (table 4).

An effect of the naphthalene acetic acid treatment is to cause an increase in the mobile auxin from the stump of the second internode, petiole, and hypocotyl.

It is possible then that the observed increase in mobile auxin from the terminal bud and hypocotyl was caused by an increased amount of mobile auxin resulting from the treatment of the first internode. These data suggest that since the auxin was moving acropetally, against the usual polar transport for auxin, it was possibly being carried in the transpiration stream. This effect of the transpiration stream on auxin transport has been observed with cuttings where

<sup>2</sup> The leaf blade was left intact—except for a narrow strip cut out along each side of the midrib and including it, from the place where the agar block was placed to the tip of the blade.

auxin at high concentrations in water solutions was applied at basal cut surfaces (3).

Experiments to determine the effect in 2 hours of naphthalene acetic acid treatment on the mobile auxin in the roots were performed by allowing the main tap root of bean seedlings to grow through the opening in the bottom of 4-inch flower pots. Seventy-two 7-day-old seedlings, grown in soil, were watered 2 hours before

TABLE 4  
AUXIN IN BEAN SEEDLINGS DIFFUSING AWAY FROM SITE OF  
2 PER CENT NAPHTHALENE ACETIC ACID  
TREATMENT IN 2 HOURS

EXPERIMENT NO.	LOCATION OF AUXIN COLLECTION	AUXIN (AS DEGREES AVERAGE NEGATIVE CURVATURE OF TWELVE AVENA COLEOPTILES)	
		UNTREATED	TREATED
69.....	Stump of second internode	$0 \pm 0$	$7.5 \pm 1.0$
71A.....	Stump of petiole	$0 \pm 0$	$5.5 \pm 2.6$
73.....	{ Stump of petiole	$0 \pm 0$	$6.3 \pm 1.4$
	{ Incision in leaf blade	$1.8 \pm 0.9$	$8.2 \pm 0.9$
	{ Stump of cotyledon	$4.2 \pm 1.6$	$6.0 \pm 1.6$
	{ Incision in hypocotyl	$1.5 \pm 1.4$	$5.1 \pm 1.2$
77.....	{ Incision in leaf blade	$5.8 \pm 1.0$	$15.5 \pm 1.3$
	{ Incision in hypocotyl	$0 \pm 0$	$4.1 \pm 2.1$

the experiment began. Half of them had the band of 2 per cent lanolin-naphthalene acetic acid around the first internode as usual. The root was cut off 1 cm. below the pots and a 3 per cent agar block,  $2 \times 2 \times 1.8$  mm., applied to the cut surface. The pot was then set over a jar containing water. Two hours later the agar blocks were removed and analyzed for auxin. No auxin was found in the blocks from the roots of the treated or untreated plants.

#### ETHER-EXTRACTABLE AUXIN

As already shown, the naphthalene acetic acid treatment increased the mobile auxin in bean seedlings. The effect of this increase on the ether-extractable growth substances was next investigated. The

treated at the middle of the first internode with 2 per cent lanolin paste of naphthalene acetic acid as before. Immediately after treatment a 3 per cent agar block ( $2 \times 2 \times 1.8$  mm.) was put at one of the following places on the plant: (*a*) on the stump of the second internode after decapitation of the terminal bud; (*b*) on the stump (about 1 mm. long) of the petiole after removal of one of the heart-shaped leaves; (*c*) on the cut end of the midrib of the leaf blade about 2 mm. beyond the junction of the petiole and blade;<sup>2</sup> (*d*) on the remaining stump of one of the cotyledons after its removal; and (*e*) on the lower half of the hypocotyl in a small incision about 1 mm. deep. The incisions into the leaf and hypocotyl were made under water to prevent air from entering the vessels. Twelve plants were used for each location of the agar blocks. The experiment was repeated for the petiole stump, leaf blade, and hypocotyl. In all cases as soon as the agar block was in position the whole plant was covered with a glass jar lined with moist paper. During days of bright sunlight the plants were shaded. The agar blocks were allowed to stay on the plants for 2 hours, the same length of time allowed for treatment in the first series of experiments. They were then tested for auxin. Generally agar blocks from comparable positions on treated and untreated plants had dried out to the same extent, so that direct comparisons of the auxin could be made. The size of the block usually did not decrease more than half. The greatest drying occurred with agar blocks applied to the hypocotyl (table 4).

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TABLE 4  
AUXIN IN BEAN SEEDLINGS DIFFUSING AWAY FROM SITE OF  
2 PER CENT NAPHTHALENE ACETIC ACID  
TREATMENT IN 2 HOURS

EXPERIMENT NO.	LOCATION OF AUXIN COLLECTION	AUXIN (AS DEGREES AVERAGE NEGATIVE CURVATURE OF TWELVE AVENA COLEOPTILES)	
		UNTREATED	TREATED
69.....	Stump of second internode	$0 \pm 0$	$7.5 \pm 1.0$
71A.....	Stump of petiole	$0 \pm 0$	$5.5 \pm 2.6$
73.....	{ Stump of petiole	$0 \pm 0$	$6.3 \pm 1.4$
	{ Incision in leaf blade	$1.8 \pm 0.9$	$8.2 \pm 0.9$
	{ Stump of cotyledon	$4.2 \pm 1.6$	$6.0 \pm 1.6$
	{ Incision in hypocotyl	$1.5 \pm 1.4$	$5.1 \pm 1.2$
77.....	{ Incision in leaf blade	$5.8 \pm 1.0$	$15.5 \pm 1.3$
	{ Incision in hypocotyl	$0 \pm 0$	$4.1 \pm 2.1$

the experiment began. Half of them had the band of 2 per cent lanolin-naphthalene acetic acid around the first internode as usual. The root was cut off 1 cm. below the pots and a 3 per cent agar block,  $2 \times 2 \times 1.8$  mm., applied to the cut surface. The pot was then set over a jar containing water. Two hours later the agar blocks were removed and analyzed for auxin. No auxin was found in the blocks from the roots of the treated or untreated plants.

#### ETHER-EXTRACTABLE AUXIN

As already shown, the naphthalene acetic acid treatment increased the mobile auxin in bean seedlings. The effect of this increase on the ether-extractable growth substances was next investigated. The

roots, hypocotyl, cotyledons, first internode, heart-shaped leaves, and terminal bud were each extracted separately 6, 20, 44, and 96 hours after the usual naphthalene acetic acid treatment. Extractions were made from the corresponding parts of untreated plants at the same times. Extractions of untreated plants were also made at the beginning of the experiment. The lanolin-naphthalene acetic acid paste was not included in the internode extractions, as the treated portion of the internode, about 4 mm. long, was cut out and only the remaining parts which had no paste on them were extracted.

The results are difficult of interpretation owing to the fact that a substance was extracted along with the auxin which caused a growth inhibition of *Avena* coleoptiles, thus rendering the standard *Avena* test for auxin inadequate, as pointed out in a previous paper (2). Positive curvatures of  $25^{\circ}$ , in  $2\frac{1}{2}$  hours after the beginning of the *Avena* test, were obtained with ether extracts of the leaves. The greatest amounts of inhibitor extracted were from the heart-shaped leaves and first internodes of 8-day-old seedlings. In the same plants the greatest amount of auxin was extracted from the terminal bud.

Extractions of the first internode of untreated and treated plants 6 hours after treatment showed respectively, 90 minutes after application of the agar blocks to the *Avena* coleoptiles, negative curvatures of  $0^{\circ}$  and  $36^{\circ}$  (calculated on 100 gm. fresh weight of material extracted; extract taken up in 0.3 cc. of 1.5 per cent agar). Sixty minutes later positive curvatures of  $29^{\circ}$  and  $9^{\circ}$ , respectively, were found. This apparent decrease in the inhibitor content of the treated internodes, as shown by the *Avena* coleoptile curvatures, was possibly caused by naphthalene acetic acid extracted from the internode masking the inhibitor present.

### Discussion

In untreated kidney bean seedlings the terminal bud is the site of the highest concentration of ether-extractable auxin but not of free-moving or mobile auxin (tables 1 and 2). Because the terminal bud is actively growing there appears to be a translocation of food materials in its direction. In *Avena* coleoptiles the tip is likewise the site of the highest concentration of ether-extractable auxin; but in contrast to the terminal bud of the bean seedling, it also produces the greatest amount of mobile auxin (3). In 6-day-old bean plants the

greatest amount of mobile auxin is produced by the heart-shaped leaves. An ether extraction of these leaves shows not only the presence of auxin but also a substance which causes positive *Avena* coleoptile curvatures. A similar situation with radish has been described (2).

In treated bean seedlings, in contrast to untreated, the first internode is a site of high concentration of both mobile and ether-extractable auxin (table 4). Whether this is mainly naphthalene acetic acid or whether it is a naturally occurring auxin has not been determined. Thus, in less than 2 hours, the naphthalene acetic acid treatment results not only in a new gradient of mobile auxin (away from the first internode), but also increases the amount of mobile auxin in the parts of the plant studied (tables 1, 3, and 4). The presence of a high content of mobile and bound auxin near the treated portion is associated with a mobilization of carbohydrate and nitrogenous materials toward that portion, and not toward the terminal bud as in the untreated plant (1).

### Summary

In 7-day-old bean seedlings, applications of a 2 per cent lanolin paste of naphthalene acetic acid applied as a band 1-2 mm. wide around the middle of the first internode caused in less than 2 hours an increase in the amount of mobile auxin in the terminal bud, internode, and hypocotyl. The treatment likewise established a new gradient of mobile auxin. It also increased the ether-extractable auxin (which includes the mobile auxin) in the first internode. Ether extractions of bean seedlings showed large amounts of auxin in the terminal bud, while in other parts of the plant a substance was found capable of causing *Avena* coleoptile growth inhibition.

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## GROWTH OF INCOMPATIBLE POLLEN TUBES IN *OENOTHERA ORGANENSIS*

STERLING EMERSON

(WITH SIX FIGURES)

### Introduction

Self-sterility is rather widely distributed among the flowering plants, and in certain instances (see 2 and 7 for references) the cytological and physiological aspects of the phenomenon have been investigated, but in no case is the underlying cause of self-sterility understood. Different plants have given rather diverse results, and *Oenothera organensis* appears to differ in several respects from other organisms so far studied. Numerous negative data relating to the physiology of self-sterility in this species have been accumulating during the last few years, which, while giving no direct lead to an attack on the problem, will be discussed briefly in part I of this paper in order to show something of the limits of the problem and to make more understandable the experiments reported here.

### I. Self-sterility in *Oenothera organensis*

#### GENETIC BASIS OF SELF-STERILITY

The way in which self-sterility is inherited in *Oenothera organensis* is identical with that originally worked out by EAST and MANGELSDORF (4) in *Nicotiana*. This so-called oppositional-factor relationship is apparently the most common basis for self-sterility in the flowering plants (7). In *O. organensis* there is a series of forty-five allelomorphs which governs the self-sterility response (5, 6, and unpublished). Two members of this series, which is commonly designated by the symbols  $S_1, S_2, S_3, \dots, S_n$ , are present in the diploid tissues of every individual, the haploid pollen and embryo sacs having one or the other in equal proportions. Incompatibility is a gametophytic character, in that the response of the pollen is determined by the particular allelomorph carried by it, with no influence from the second allelomorph present in the parental sporophyte. Pollen carrying any particular allelomorph, say  $S_i$ , fails to produce normally

developing pollen tubes in any style in which the same allelomorph is present,  $S_1/S_2$ ,  $S_1/S_3$ , . . . .  $S_1/S_n$ , but does produce functioning pollen tubes in any style not carrying that particular allelomorph,  $S_2/S_3$ ,  $S_7/S_{26}$ , etc. Hence crosses between different individuals may behave in any of three distinct ways: (1) in crosses between plants of identical constitutions, such as  $S_1/S_2 \times S_1/S_2$ , neither  $S_1$  nor  $S_2$  pollen can grow in the  $S_1/S_2$  style and the combination is completely cross-sterile; (2) in crosses between plants having one allelomorph in common, as in  $S_1/S_2 \times S_1/S_3$ , pollen carrying  $S_1$  fails to function in the  $S_1/S_2$  style but  $S_3$  pollen develops normally, its nuclei eventually combining with the two sorts of eggs to give seeds of the constitution  $S_1/S_3$ , identical with the pollen parent, and  $S_2/S_3$ , which is unlike either parent; (3) in crosses such as  $S_1/S_2 \times S_3/S_4$ , in which the parents have no allelomorph in common, both  $S_3$  and  $S_4$  pollens function to give seeds of four different constitutions— $S_1/S_3$ ,  $S_2/S_3$ ,  $S_1/S_4$ , and  $S_2/S_4$ , none of which is identical with either parent. This scheme of inheritance has been established for *O. organensis* both by progeny tests and by direct observation of pollen-tube behavior within the tissues of the styles (5).

#### POLLEN-TUBE GROWTH IN STIGMA AND STYLE

On the stigma, both compatible and incompatible pollens (having respectively neither or one of the allelomorphs present in the pistillate parent) germinate within 15 minutes. At first there is no definitely distinguishable difference between the two sorts of pollen tubes, but later, generally within the first hour after pollination, the incompatible tubes cease growing and remain short while the compatible tubes continue to grow down through the style to the ovary.

As the compatible pollen tubes grow down the style, the entire cell contents pass down the tubes, leaving the pollen grains empty and shrunken. Incompatible pollen tubes ordinarily do not grow sufficiently long to empty their cell contents. This distinction between compatible and incompatible pollen becomes evident within 3 hours after pollination. Photomicrographs of this condition have been published (5, figs. 3-6).

The growth rate of compatible pollen tubes is constant (5) and at ordinary temperatures is in the neighborhood of 6-8 mm. per

hour. Compatible pollen tubes generally reach the ovary, a distance of 150–180 mm. in most flowers,<sup>1</sup> within 24 hours after pollination.

The maximum growth attained by incompatible pollen tubes may vary (*a*) with the season, (*b*) among different flowers from a single plant, (*c*) among different plants of the same constitution, and (*d*) according to the allelomorph present. Seasonal variation will be discussed later, in the section on “end-season fertility.” Examples of variation among flowers from the same plant can be found in the controls in tables 4 and 5. Among plants carrying the same self-sterility allelomorphs some may show much more variability than others. These differences are perhaps due to genetic dissimilarities not associated with the self-sterility allelomorphs, and the effects may be very indirect. For the most part, variation in the response of incompatible pollen tubes is not sufficiently great to obscure the distinction between compatible and incompatible tubes if they have been allowed to grow 4 hours or longer. The chief exceptions to this condition are the responses of self-sterility allelomorphs  $S_9$  and  $S_{39}$ .

In the seasons of 1937 and 1938, considerable difficulty was experienced in identifying allelomorph  $S_9$ . In many styles carrying this allelomorph, pollen tubes also carrying  $S_9$  grew long enough to empty the pollen grains of their cell contents and make them indistinguishable from compatible pollen grains. In other styles from the same plants, however, pollen carrying  $S_9$  gave the normal incompatible response. In the current season (1939) this difficulty largely disappeared, perhaps owing to changed environmental conditions—but equally likely to an increased familiarity with the material on my part, which enabled a clearer distinction to be made between the response of allelomorph  $S_9$  and compatible allelomorphs.

The incompatible response of allelomorph  $S_{39}$  is still more extreme than that of  $S_9$ . At the height of the flowering season it is common for at least part of the tubes carrying  $S_{39}$  to grow as rapidly in incompatible styles as in compatible styles for at least 10 hours after pollination. Direct examinations of pollen-tube growth within the

<sup>1</sup> Growth of pollen tubes is not measurably different in flowers removed from the plant from that in flowers left attached. Unless otherwise noted, the procedure followed was to remove the flowers before pollination and store them in moist chambers during the interval in which the pollen tubes were allowed to grow.

styles were not made after longer intervals, but whether or not the pollen tubes reached the ovaries, no seeds set following self-pollination, indicating that  $S_{39}$  is also a sterile allelomorph. Flowers were heavily pollinated (500-1000 pollen grains) about 8 hours before normal anthesis and again 24 hours later, the second pollination coinciding with the time at which pollinations are made for the usual tests. No seeds were obtained from twenty-seven such pollinations,

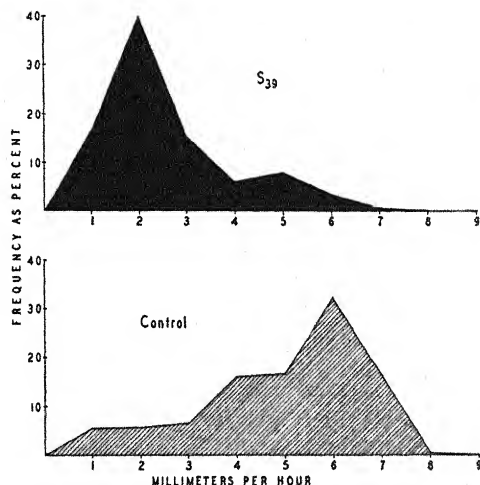


FIG. 1.—Frequency distribution of pollen tubes having different growth rates. Upper curve represents growth of pollen carrying  $S_{39}$  in incompatible styles (based on measurements of 1888 pollen tubes made 3-10 hours after pollination, in 25 different styles). Lower curve represents growth of compatible pollen tubes in styles of same plants (measurements of 641 tubes in 22 flowers at equivalent times).

whereas every flower pollinated in the same manner—but with compatible pollen—has given a good set of seeds during the current season. When flowers heterozygous for  $S_{39}$  are self-pollinated, nearly all pollen tubes carrying that allelomorph grow sufficiently long to empty the pollen grains of their contents, giving an appearance exactly similar to that found when a pollination is made between two flowers having one allelomorph in common.<sup>2</sup> But comparatively

<sup>2</sup> The plant used in this instance was  $S_7/S_{39}$ . Crosses using this plant as pollen parent showed that  $S_7$  gave the typical short tube, incompatible response in all styles also carrying  $S_7$ ; similarly, pollen carrying  $S_7$  from other sources always gave the typical short-tube response in styles of  $S_7/S_{39}$ . The atypical response of half the pollen tubes in styles of  $S_7/S_{39}$  following self-pollinations must therefore be attributed to  $S_{39}$ .

few of the tubes carrying  $S_{39}$  grow as rapidly as compatible tubes, giving a different distribution of tubes within the style (fig. 1).

In certain self-sterile plants (for example in *Abutilon*; 7, pl. I, fig. 2) incompatible pollen tubes become swollen at the tips, or burst. This condition does not exist regularly in *Oenothera organensis*. In occasional flowers pollen tubes with swollen tips have been observed, but these have nearly always been found following completely compatible pollinations. This condition in *O. organensis* must be attributed to some peculiarity of the style of the particular flowers in which it occurs, since the pollen has reacted normally in other styles. Swollen pollen tubes have been observed more frequently in flowers from plants grown in the greenhouse during the winter than from field-grown plants.

Three-quarters of an hour after pollination, when compatible and incompatible pollen tubes are of similar lengths and near the maximum length of incompatible tubes, there is no striking difference between them. In some compatible tubes the starch grains appear to be digested to a greater extent at this time, but there is considerable variation from one tube to another and the difference has not been established.

#### BUD FERTILITY AND END-SEASON FERTILITY

In many self-sterile plants (such as *Nicotiana*, 1) seeds may be obtained following self-pollinations provided either (a) the stigmas are pollinated long before the flowers would normally open (bud fertility), or (b) the flowers have been produced at a time when the plant has nearly stopped flowering (end-season fertility). Neither of these types of so-called pseudo-fertility occurs in *O. organensis*. The stigmas of this species are not receptive to pollen much earlier than 36 hours before normal anthesis. If they are pollinated earlier the pollen fails to germinate, and whenever the stigma is receptive both compatible and incompatible pollens respond as on open flowers. When plants are at the end of their flowering period the tubes from incompatible pollen often grow longer than at the height of the flowering season, but no seed has ever been obtained following self-pollination under these conditions. *O. organensis* has proved to be one of the most completely self-sterile species so far investigated. None of the methods by which seeds from self-pollinations may be obtained in other species is effective in this one.

## LOCALIZED INHIBITORY ZONES

EAST (3) suggested, on the basis of growth rates of compatible and incompatible pollen tubes in immature styles—as contrasted with that of compatible pollen tubes in mature styles—that there is a localized zone near the base of the stigma in *Nicotiana* within which inhibition of incompatible pollen tubes takes place. SEARS (7) found that the block to the growth of incompatible pollen tubes in *Bras-*

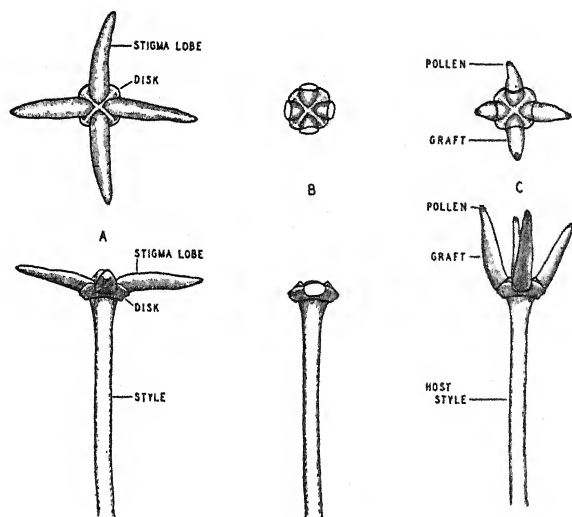


FIG. 2.—Method of making stigma grafts. Compatible pollen is placed on tips of grafted stigma lobes and tube growth measured below graft in either compatible or incompatible tissue, as the case may be.

*sica oleracea* var. *italica* was at the surface of the stigma. If the surface was removed, incompatible tubes grew nearly as well as compatible, giving nearly as good a set of seeds.

No such localized block seems to be present in *Oenothera organensis*. Whenever accurate measurements of pollen-tube length are to be made, it is customary to cut off the four lobes of the stigma (as in B, fig. 2) and pollinate the more-or-less hemispherical surface remaining, so that all tubes have the same distance to grow before entering the style. Incompatible pollen tubes grow no longer from the cut surfaces than from the intact surfaces of such stigmas. The results of certain style and stigma grafts bear on this point.

Stigma grafts were made in the manner illustrated in figure 2. The four lobes of the stigma of one constitution, say  $S_1/S_2$ , were cut off and the stigma lobes of a different constitution,  $S_3/S_4$ ,

TABLE 1

GROWTH OF POLLEN TUBES IN COMPATIBLE AND INCOMPATIBLE TISSUES  
AFTER PASSING THROUGH COMPATIBLE STIGMA GRAFTS (FIG. 2)

STYLE	GRAFT	POLLEN	HOURS AFTER POLLI- NATION	LENGTHS OF POLLEN TUBES BELOW GRAFT (MM.)												MEAN LENGTH PER HOUR (MM.)
				0-1- 10	10- 20	20- 30	30- 40	40- 50	50- 60	60- 70	70- 80	80- 90	90- 100	100- 110	110- 120	
$S_1/S_2$ ....	$S_3/S_4$	$S_1/S_2$	8	3	..	..	..	..	..	..	..	..	..	..	..	0.2
			10	3	..	1	..	..	..	..	..	..	..	..	..	0.9
			20	3	2	..	..	..	..	..	..	..	..	..	..	0.4
$S_3/S_4$ ....	$S_3/S_4$	$S_1/S_2$	8	..	..	..	4	..	..	..	..	..	..	..	..	4.4
			20	1	..	..	..	..	..	..	..	3	..	1	1	3.9
			..	..	..	..	..	..	..	..	..	..	..	..	..	..
$S_3/S_4$ ....	$S_1/S_2$	$S_3/S_4$	8	{	3	..	..	..	..	..	..	..	..	..	..	0.2
			..	{	3	..	..	..	..	..	..	..	..	..	..	0.1
			10	{	..	..	1	1	..	..	..	..	..	..	..	3.0
			..	{	1	1	1	..	..	..	..	..	..	..	..	1.2
			20	4	..	..	..	..	..	..	..	..	..	..	..	0.2
			..	..	..	..	..	..	..	..	..	..	..	..	..	..
$S_1/S_2$ ....	$S_1/S_2$	$S_3/S_4$	8	{	..	1	3	..	1	..	..	..	..	..	..	3.5
			..	{	..	..	..	1	2	1	..	..	..	..	..	6.1
			10	{	2	..	..	3	5	3	..	..	..	..	..	4.5
			..	{	..	1	1	2	..	..	1	..	..	..	..	3.7
			20	{	3	..	..	2	1	3	1	..	..	..	..	1.7
			..	{	..	..	..	1	1	1	2	3	..	2	..	3.4
$S_1/S_2$ ....	None	$S_1/S_2$	8	50	7	..	..	..	..	..	..	..	..	..	..	0.3
			10	47	14	5	2	1	..	..	..	..	..	..	..	0.9
			20	55	1	..	..	..	..	..	..	..	..	..	..	0.3
$S_3/S_4$ ....	None	$S_3/S_4$	8	62	..	..	..	..	..	..	..	..	..	..	..	0.4
			10	54	1	..	..	..	..	..	..	..	..	..	..	0.5
			20	32	..	..	..	..	..	..	..	..	..	..	..	0.3

were grafted on in their places. If the cuts are cleanly made with a sharp blade the stigma lobes to be grafted on adhere strongly when the cut surfaces are brought in contact and no adhesive has been used. The grafted stigma lobes were pollinated at their tips with pollen of the same constitution as the host stigma,  $S_1/S_2$  in this

instance, which is compatible with the tissues above the graft union and incompatible with those below. As a control, stigma lobes  $S_3/S_4$  were grafted on to host stigmas of the same constitution and again pollinated by  $S_1/S_2$ , which is compatible with the tissues below the graft union as well as above. The data from one such experiment are summarized in table 1.

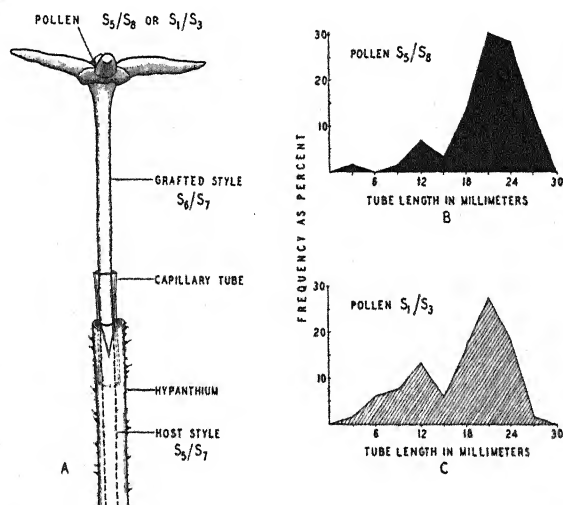


FIG. 3.—Pollen-tube growth in grafted styles. A, method of making grafts; B, distribution of pollen tubes in compatible grafted styles when host style is incompatible with one of allelomorphs carried in pollen; C, distribution of pollen tubes in compatible grafted styles when host style is compatible with both allelomorphs.

Six of the eight test grafts gave approximately as little tube growth below the graft unions as the incompatible controls, whereas each of the eight control grafts (compatible) showed much greater growth. In two of the test grafts the incompatible tubes grew at nearly the rate of compatible tubes in the control grafts, but in other experiments with the same plants incompatible pollinations sometimes gave as much growth, and it is thought that there was no effect of grafting in this experiment.

Grafts of a similar sort were made at varying distances down the style. These were made essentially as illustrated in figure 3A, but the grafts were made after the stigmas had been pollinated, at a time when the longest tubes were nearly at the level to be cut. In

these grafts comparatively few pollen tubes cross the graft unions, but the few data obtained were consistent with the interpretation that the incompatibility response takes place at any level in the stigma or style.

In another experiment stigma lobes of one constitution,  $S_1/S_2$ , were grafted directly on to the upper surfaces of ovaries of another constitution,  $S_3/S_4$ , and pollinated with pollen ( $S_3/S_4$ ) which was compatible with the grafted stigma lobes but incompatible with the ovaries. Although many attempts were made, no seed was obtained from pollinations of this sort, indicating that the incompatibility response may occur in the ovary also.

These experiments indicate that the incompatibility response is not confined to any particular zone of the style or stigma in *O. organensis*. This conclusion is also borne out by the behavior of pollen tubes carrying allelomorph  $S_{39}$ , already referred to, and by the behavior of incompatible pollen tubes in styles from plants kept in the dark, to be discussed in the second part of this paper, in both of which the response is so weakened that tubes grow to considerable lengths before their growth is stopped.

These experiments also support the conclusion, previously stated by EAST and others, that incompatible pollen tubes are inhibited in some way upon coming in contact with the tissues of the stigma or style, rather than that compatible pollen tubes receive some necessary growth stimulus from compatible tissues. If the latter alternative were true, pollen tubes in the experiments just described should continue rapid growth in the incompatible tissues below the graft unions as long as cytoplasm remained in the portions of the tubes above the graft unions where they were in direct contact with compatible tissues. This is not the case.

#### DIFFUSIBLE SUBSTANCES

From experiments with *Petunia violacea*, YASUDA (8, 9, 10) concluded that substances diffusing from the placental region of the ovary controlled the growth of compatible and incompatible pollen tubes within the styles. He made various style grafts and found that the response of the pollen tubes was always that to be expected from the genetic constitution of the ovaries, regardless of the constitution of the styler tissues through which the tubes were growing.

Pollen tubes grown in vitro showed the same response when pieces of placental tissues were added to the solutions.

Similar style grafts were made with *O. organensis*. In my material, however, the response of pollen tubes always depended upon the constitution of the tissues through which they were growing, with no noticeable effect from the ovary. Grafts were made as indicated in figure 3A. The hypanthium and style were cut about 50 mm. below the stigma. The cut ends of the host style and of the

TABLE 2

GROWTH OF POLLEN TUBES IN GRAFTED STYLES. ALL GRAFTS WERE  $S_6/S_7$  ON TO  $S_5/S_7$  STYLES (FIG. 3). THE PLUS SIGN INDICATES A COMPLETELY COMPATIBLE COMBINATION; THE FRACTION  $\frac{1}{2}$ , A COMBINATION IN WHICH HALF THE POLLEN IS COMPATIBLE AND HALF INCOMPATIBLE

POLLEN	REACTION		FREQUENCIES OF TUBES	
	GRAFT	HOST	0.1-15 MM.	15.1-30 MM.
$S_7/S_3$ .....	+	+	$\begin{Bmatrix} 12 \\ 11 \end{Bmatrix}$	$\begin{Bmatrix} 16 \\ 26 \end{Bmatrix}$
$S_5/S_8$ .....	+	$\frac{1}{2}$	$\begin{Bmatrix} 8 \\ 7 \\ 6 \\ 5 \\ 3 \\ 8 \end{Bmatrix}$	$\begin{Bmatrix} 51 \\ 47 \\ 30 \\ 48 \\ 32 \\ 40 \end{Bmatrix}$

style to be grafted on to it were brought in contact inside a capillary glass tube of suitable diameter, which was wedged into the hypanthium for support. The whole flower, still attached to the plant, was covered with a small moist chamber and allowed to stand overnight. Grafted stigmas which showed no signs of wilting after 14 hours were pollinated at the base of the stigma lobes, and the lengths of pollen tubes in the grafted style were determined 4 hours later. In the controls, pollen completely compatible with both grafted and host styles was used so that there should be no inhibition, either from the tissues in which the tubes grew or from substances diffusing from the ovary. The pollen used was also com-

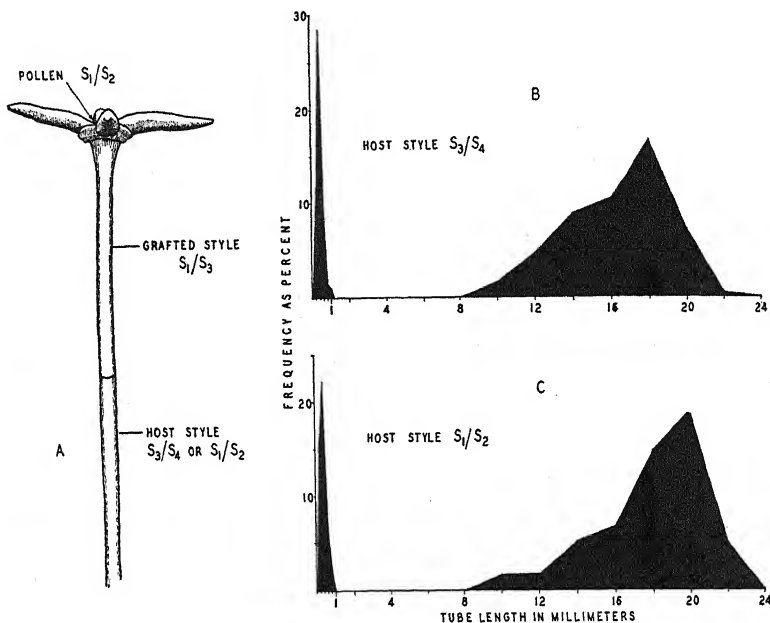


FIG. 4.—Pollen-tube growth in grafted styles. A, method of grafting; B, distribution of tubes of which half are compatible, half incompatible with grafted style in which they were growing, when host style is compatible with both types of pollen; C, distribution of tubes under similar conditions except that host style is incompatible with both types of pollen.

TABLE 3

GROWTH OF  $S_1/S_2$  POLLEN IN  $S_1/S_3$  STYLES WHICH  
HAD BEEN GRAFTED ON TO STYLES OF OTHER  
CONSTITUTIONS 14 HOURS EARLIER

HOST STYLE	POLLEN TUBES (1 MM. OR LESS)	POLLEN TUBES (10 MM. OR MORE)
$S_3/S_4$ .....	$\begin{cases} 28 \\ 30 \\ 25 \end{cases}$	$\begin{cases} 25 \\ 24 \\ 47 \end{cases}$
$S_1/S_2$ .....	$\begin{cases} 33 \\ 33 \\ 31 \end{cases}$	$\begin{cases} 31 \\ 31 \\ 34 \end{cases}$

pletely compatible with the grafted style, but only half the pollen (carrying  $S_2$ ) was compatible with the host style, the other half ( $S_3$ ) incompatible, so that none of the tubes should be inhibited by tissues in which they were growing, but half of them should be inhibited if controlling substances diffused from the ovary. The data presented in table 2 and figure 3*B, C* show that there is no greater tendency toward a bimodal distribution of tubes (compare with distributions illustrated in figure 4*B, C*) in the tests than in the controls, indicating that there is no inhibiting effect from substances diffusing from below the graft.

In another experiment the growth of  $S_1/S_2$  pollen tubes was followed in  $S_1/S_3$  styles which had been grafted on to other styles (fig. 4). With no effect from grafting,  $S_1$  pollen should be incompatible and  $S_2$  pollen compatible with the tissues in which they were growing. In grafts on to  $S_3/S_4$  the compatible tubes should be unaffected, but the incompatible tubes,  $S_1$ , might be stimulated in growth by substances diffusing from compatible tissues below the graft. In grafts on to  $S_1/S_2$  the incompatible tubes should be unaffected, whereas the compatible tubes,  $S_2$ , might be inhibited by substances diffusing from the incompatible tissues below the graft. The data presented in table 3 and figure 4*B, C* again indicate that there is no observable influence from substances diffusing from below the graft.

To test for substances which might diffuse throughout the plant at very slow rates, root grafts similar to that illustrated in figure 5 were made. On two such grafts adventitious buds developed on both

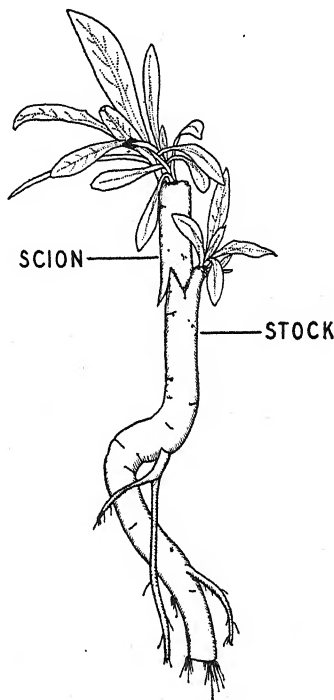


FIG. 5.—Root graft with adventitious shoots on both stock and scion.

stock and scion, and flowers were obtained on both simultaneously. Every test indicated that pollen-tube response was determined by the tissues in which the tubes were growing, with no effect from the other part of the graft. For example, a flower on the scion  $S_2/S_4$  was pollinated with pollen from the stock  $S_3/S_4$ : half the pollen tubes ( $S_4$ ) grew to less than 1 mm. in  $4\frac{1}{2}$  hours and half ( $S_3$ ) grew to 30 mm. (maximum), showing no inhibition from the stock. Self-pollinations showed that both stock (tube growth to 2 mm.) and scion (tube growth to 1.5 mm.) remained completely self-sterile. In these instances there was good union between stock and scion, and if inhibitory or stimulating substances diffused readily throughout the plant they should have been detected.

## II. Effect of light on pollen-tube growth

When plants were grown in the greenhouse in the winter and flowered by artificial lighting, there was occasionally a less sharp distinction between the growth of compatible and incompatible pollen tubes within 4 hours after pollination than is generally the case with field-grown plants. This type of response was most noticeable after a period of cloudy weather, and it was thought that the decreased light intensity might be the cause. A test of the effect of light on pollen-tube growth was made with flowers from field-grown plants in the summer of 1939.

For the principal experiment a clone of three plants of constitution  $S_{12}/S_{13}$  was used. These plants were chosen because they normally flowered abundantly over a long season and because the response of incompatible pollen tubes following self-pollination was extremely regular—in nearly all flowers tested the incompatible tubes had a maximum length of 3 mm. or less.

Each of the three plants was covered in turn by a dark-house made of ply-wood, measuring 3 feet by 3 feet by 5 feet, painted black on the inside and white on the outside and ventilated top and bottom. A small amount of light diffused through the ventilators and enough light to see by was admitted when the flowers were being emasculated. Plants could be kept in the dark for 7–8 days, after which all remaining buds fell off and no further flowers would be produced for 3 weeks or longer after returning the plant to the light.

Control data were collected from each plant before it was placed in the dark and from other plants of the clone during the time one plant was in the dark. Since the controls from the three plants were uniform throughout, they are not presented separately in table 4. The tests were run so as to distinguish between possible effects of continued darkness on the pollen and stigmas separately. That is, some stigmas grown in the dark were pollinated with pollen also from the plant in the dark and others with pollen from a member of the clone kept in the light; similarly, some stigmas from a plant in the light were pollinated with pollen from the same plant and some with pollen from the plant kept in the dark. No measurable change was observed in the pollen of plants kept in the dark, even after 7 days, at which time it gave the characteristic short-tube growth in incompatible styles kept in the light, while in compatible pollinations it produced tubes of the same length as compatible pollen from plants kept in the light. Since the source of pollen made no difference in the growth response, the different types of pollinations will not be presented separately.

The styles of flowers from plants kept in the dark showed no change as measured by the response of compatible pollen tubes. Following pollination by  $S_{16}/S_{17}$ , the growth of pollen tubes was at the same rate in styles from plants kept in the dark as from plants kept in the light. On the other hand, styles from plants kept in the dark did show a marked change as measured by the response of incompatible pollen tubes (table 4).

No noticeable change occurs in the first 48 hours that the plant is kept in the dark. After 72 hours, however, there is a gradual increase in the length of incompatible tubes, which reaches an apparent maximum at about 144 hours. The first signs of etiolation of the young shoots generally appear after 96 hours. After 144 hours most flowers are abnormal in appearance, since the different floral parts have not grown at the usual comparative rates. All parts of the flower are considerably shortened, but especially the hypanthium, so that the style and stigma often project far above the sepal tips. In the more abnormal flowers abscission layers seem to have been formed at the top of the ovary, causing the hypanthia and styles

TABLE 4  
FREQUENCIES OF STYLES WITH POLLEN TUBES OF DIFFERENT  
MAXIMUM LENGTHS (INCOMPATIBLE POLLINA-  
TIONS ON  $S_{12}/S_{13}$ )

LENGTH (MM.)	HOURS IN DARK						
	0	24	48	72	96	120	144-192
0.5.....	61	14	8	3	.....	.....	2
1.0.....	82	10	7	6	3	.....	1
1.5.....	55	9	6	20	6	1	.....
2.0.....	31	3	5	20	12	.....	.....
3.0.....	12	.....	3	6	13	2	1
4.0.....	5	.....	.....	5	7	5	.....
5.0.....	3	.....	.....	1	5	8	.....
6.0.....	6	.....	.....	.....	3	6	.....
7.0.....	3	.....	.....	.....	2	4	.....
8.0.....	.....	.....	.....	.....	.....	1	2
9.0.....	.....	.....	.....	.....	1	1	2
10.0.....	.....	.....	.....	.....	.....	9	.....
11.0.....	.....	.....	.....	1	.....	3	3
12.0.....	2	.....	.....	2	.....	8	3
13.0.....	.....	.....	.....	.....	.....	4	2
14.0.....	.....	.....	.....	1	.....	1	4
15.0.....	.....	.....	.....	.....	.....	1	3
16.0.....	.....	.....	.....	.....	.....	.....	3
17.0.....	.....	.....	.....	.....	.....	.....	2
18.0.....	.....	.....	.....	.....	.....	.....	3
19.0.....	.....	.....	.....	.....	.....	1	1
20.0.....	.....	.....	.....	.....	.....	.....	1
21.0.....	.....	.....	.....	.....	.....	.....	1
22.0.....	.....	.....	.....	.....	.....	1	.....
23.0.....	.....	.....	.....	.....	.....	.....	.....
24.0.....	.....	.....	.....	1	.....	.....	.....
25.0.....	.....	.....	.....	.....	.....	1	1
26.0.....	.....	.....	.....	.....	.....	.....	.....
27.0.....	.....	.....	.....	.....	.....	1	.....
28.0.....	.....	.....	.....	.....	.....	.....	.....
29.0.....	.....	.....	.....	.....	.....	.....	.....
30.0.....	.....	.....	.....	.....	.....	.....	.....
31.0.....	.....	.....	.....	.....	.....	.....	1
32.0.....	.....	.....	.....	.....	.....	.....	.....
33.0.....	.....	.....	.....	.....	1	.....	.....
Mean length..	1.6	1.0	1.3	2.9	3.8	9.3	13.5

to break off at this level. Many of the styles with very short tubes after 144 hours or longer in the dark were from abnormal flowers.

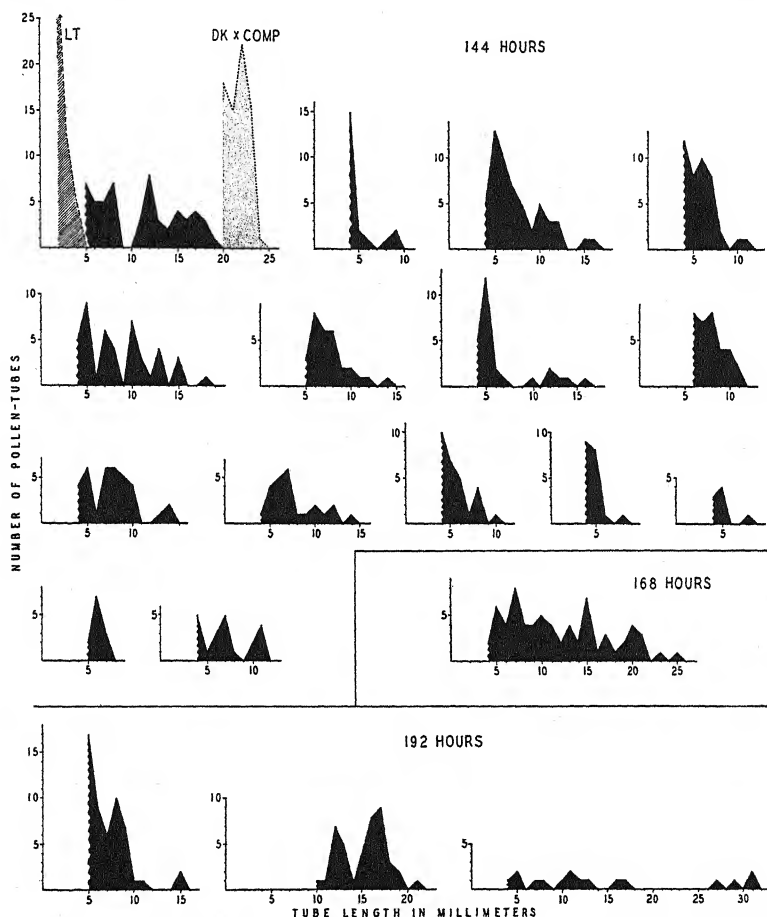


FIG. 6.—Distributions (in absolute numbers) of pollen tubes of different length, within styles of plant of  $S_{12}/S_{13}$  clone. Solid black curves show distributions of incompatible pollen tubes in styles of different flowers from plant kept in dark for times indicated. Lightly shaded curve (LT in upper left figure) shows greater-than-average growth of incompatible pollen tubes in style from plant kept in light (also  $S_{12}/S_{13}$ ); stippled curve (DK  $\times$  COMP in same figure) shows distribution of compatible ( $S_{16}/S_{17}$ ) pollen tubes in style ( $S_{12}/S_{13}$ ) from plant kept in dark.

The data presented in table 4 show the measurements of only the longest pollen tube in each style. From 72 hours on, these may

be as long in some styles as compatible tubes grown for the same length of time ( $4\frac{1}{2}$  hours—compatible pollen tubes generally grow 25–30 mm. in that time). But in compatible pollinations most of the pollen tubes are nearly as long as the longest, whereas comparatively few incompatible pollen tubes approach the longest in these instances. The distribution curves in figure 6 illustrate this point. These curves are based on measurements of tubes 4 mm. or longer, but since care was taken to pollinate all stigmas equally (about 300 pollen grains per stigma), the data from the different flowers should be roughly comparable.

Other plants of different constitutions also showed the same response to long periods in the dark. These plants, however, showed much greater variability between individual flowers when kept in the light, and the results are not so striking. One such example is summarized in table 5.

#### TESTS FOR SEED SET

The greater growth of incompatible pollen tubes in styles from plants kept in the dark is apparently due to a weakening of the incompatibility response and not to its complete disappearance. Incompatible pollinations were made as follows: six after 96 hours in the dark, four after 120 hours, nine after 144 hours, and ten after 192 hours, some being pollinated as open flowers, some as buds. Not one seed set following these twenty-nine pollinations. Control pollinations with compatible pollen were not made, so the possibility remains that failure of seed set could be due to the general weakening of the plant. In one experiment pollen tubes were allowed to grow for 8 hours instead of the usual  $4\frac{1}{2}$  hours before examination, and while the experiment was not adequately controlled, the results indicated that there was little more growth in 8 hours than in  $4\frac{1}{2}$ , leading to the inference that the pollen tubes never reached the ovaries.

#### DIFFUSIBLE SUBSTANCES

The distal three-quarters of about ten branches of one plant of the  $S_{12}/S_{13}$  clone were inclosed in a dark chamber, the remainder of the plant being exposed to light. Under these conditions the

TABLE 5  
 FREQUENCIES OF STYLES WITH POLLEN TUBES OF  
 DIFFERENT MAXIMUM LENGTHS (INCOMPATIBLE  
 POLLINATIONS ON S<sub>2</sub>/S<sub>3</sub>)

LENGTH (MM.)	HOURS IN DARK									
	0	24	48	72	96	120	144	168	192	216
0.5.....	3	8	3	9	.....	4	3	1	.....	.....
1.0.....	6	3	2	8	1	1	.....	3	.....	.....
1.5.....	5	3	3	5	6	4	1	.....	.....	.....
2.0.....	6	.....	2	8	2	1	3	.....	.....	1
3.0.....	6	.....	1	2	.....	1	.....	.....	.....	.....
4.0.....	7	.....	2	1	2	.....	2	.....	.....	.....
5.0.....	5	.....	1	.....	.....	1	.....	1	.....	.....
6.0.....	5	.....	1	.....	.....	.....	4	6	.....	1
7.0.....	5	.....	.....	.....	2	1	2	1	.....	.....
8.0.....	1	.....	.....	1	.....	2	.....	1	.....	.....
9.0.....	1	.....	.....	.....	.....	.....	1	2	.....	.....
10.0.....	2	.....	.....	.....	.....	.....	.....	2	.....	.....
11.0.....	.....	.....	.....	.....	.....	1	1	1*	.....	1
12.0.....	.....	.....	.....	.....	1	.....	2	.....	.....	2
13.0.....	.....	.....	.....	.....	.....	.....	.....	2	1	1
14.0.....	1	.....	1	.....	.....	.....	1	.....	1	1
15.0.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
16.0.....	.....	.....	.....	.....	.....	.....	.....	1*	.....	.....
17.0.....	1+1*	1	.....	.....	.....	.....	.....	.....	.....	.....
18.0.....	.....	.....	.....	.....	.....	.....	1	.....	.....	1
19.0.....	1	.....	.....	.....	.....	.....	.....	.....	1	1
20.0.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
21.0.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	1
22.0.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
23.0.....	.....	.....	.....	.....	.....	.....	.....	1	.....	.....
24.0.....	.....	.....	.....	.....	.....	.....	.....	1*	1*	1
25.0.....	.....	.....	.....	.....	.....	.....	.....	1*	.....	.....
26.0.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	1
32.0.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	1*
45.0.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Mean length	4.9	1.7	3.1	1.6	3.0	2.8	7.0	9.6	18.0	17.7

\* Abundant pollen-tube growth resembling compatible pollinations.

branches kept in the dark flowered almost indefinitely, but without giving any increased growth of incompatible pollen tubes (table 6).

Another plant (constitution  $S_5/S_7$ ) was kept in the dark for 120 hours, after which most of the plant was exposed to the light, with a few branches continued in the dark. The dark effect was still pronounced after the plant had been returned to the light for 24 hours, but after 48 hours the incompatibility response was back to normal. Unfortunately there were almost no flowers produced on

TABLE 6  
FREQUENCIES OF STYLES WITH POLLEN TUBES OF DIFFERENT  
MAXIMUM LENGTHS (INCOMPATIBLE  
POLLINATIONS ON  $S_{12}/S_{13}$ )

LENGTH (MM.)	BRANCHES IN LIGHT	BRANCHES IN DARK (TIME IN HOURS)							
		24	72	120	144	168	192	216	288
0.5 .....	19	3	3	.....	.....	1	1	3	.....
1.0 .....	9	.....	.....	1	.....	.....	.....	.....	4
1.5 .....	3	1	1	.....	.....	.....	1	.....	1
2.0 .....	1	.....	.....	3	.....	.....	.....	.....	.....
3.0 .....	2	.....	.....	.....	1	.....	.....	.....	.....
4.0 .....	.....	.....	.....	.....	.....	.....	.....	.....	.....
5.0 .....	1	.....	.....	.....	.....	.....	.....	.....	.....
6.0 .....	1	.....	.....	.....	.....	.....	.....	.....	1
Mean length..	1.2	1.4							

the branch continued in the dark after the first day, but recovery was apparently as rapid as on the branches in the light (table 7).

It has been shown in part I of this paper that the specific inhibitory factors responsible for the incompatibility reaction do not diffuse from tissues of one constitution to those of another. The two experiments just described indicate that substances diffusing from branches kept in the light prevent branches kept in the dark from showing the typical dark reaction. It is probable that these substances are not the specific incompatibility factors, since these are known not to diffuse readily. It is possible that some general precursor of all specific factors is made only in sunlight, but it is equally

TABLE 7

FREQUENCIES OF STYLES WITH POLLEN TUBES OF DIFFERENT  
MAXIMUM LENGTHS (INCOMPATIBLE POLLINA-  
TIONS ON  $S_5/S_7$ )

LENGTH (MM.)	ENTIRE PLANT IN DARK (TIME IN HOURS)					AFTER 120 HOURS IN DARK					
						BRANCHES RE- TURNED TO LIGHT (TIME IN HOURS)			BRANCHES RE- TAINED IN DARK (TIME IN HOURS)		
	24	48	72	96	120	24	48	72	24	48	72
0.5	2						2	2		1	
1.0	7	2	1				6	4			
1.5	5	3			2		5	5			
2.0	4	4	2	1			3	4			
3.0	2	2	5	1		2	1	4			1
4.0		1	1			3		1			
5.0		1		2	1	2					
6.0				1	2	3			2		
7.0		1	2	3		1			1		
8.0						1					
9.0			1	3	1	3			1		
10.0				1	1						
11.0			2		3						
12.0				1	1	2					
13.0			2								
14.0				2					1		
15.0					1						
16.0		1		1							
17.0				1							
18.0				1							
19.0											
20.0				2							
21.0				2							
25.0			1								
Mean length.	1.5	3.5	7.0	11.5	8.3	6.6	1.4	1.8	8.5	1.8	

possible that the dark reaction is due to a general physiological weakening of the plant, which is overcome by exposing part of the plant to light. Unfortunately the root grafts described earlier had been discarded, so that it has not yet been possible to test directly for the specificity of the dark reaction.

Buds removed individually from the plant in the dark and kept for 24 hours in a dark moist chamber, either in water or in a 2 per cent sugar solution, continued to show the typical dark reaction. If as little as 1 per cent (by dry weight) of ground-up leaves was added either to the water or sugar solution, the buds wilted and failed to open. When stigmas of these wilted flowers were pollinated, the pollen-tube growth was atypical, the tubes having nearly twice the diameter of tubes in fresh styles. Flowers on cut branches reacted in the same way to the addition of leaf extract.

### Summary

1. The characteristic features of self-sterility in *Oenothera organensis* are discussed. The genetic basis of self-sterility is a series of some forty-five allelomorphs of one gene. The reaction of the pollen is a gametophytic character, governed solely by the genetic constitution of the pollen itself, with no influence from the second allelomorph carried by the sporophyte plant on which it is borne. Incompatible pollen tubes differ from compatible in extent of growth, and in no other discernible morphological character. Self-sterility is apparently completely effective in preventing seed formation in this species: there is no bud fertility or end-season fertility and all usual methods of obtaining self-pollinated seeds have failed. The incompatibility response is due to an inhibition of pollen-tube growth, which is not governed by diffusible substances but seems to be a reaction between the pollen tube and the tissue in which it is growing.

2. The extent of growth of incompatible pollen tubes varies with the particular sterility allelomorph present in the pollen, with other undetermined genetic factors, and with certain environmental conditions. The most pronounced effect of changes in environment is that resulting from prolonged absence of light. When plants have

been kept in the dark for 3 days or longer, their styles permit much longer incompatible tube growth than occurs in flowers from plants kept in the light. The maximum effect is noticed after 5 days, after which the plant develops signs of general weakness and usually stops flowering after the 7th or 8th day. When parts of the plants are covered and the remainder left exposed to light, flowers from branches kept in the dark do not permit increased growth of incompatible pollen tubes. It has not been determined that the diffusion from branches in the light to those in the dark involves substances specifically connected with the incompatibility reaction. It is equally likely that the greater growth of incompatible pollen tubes in flowers from the dark is due to a general physiological debility of the plant, which can be overcome by leaving part of the plant exposed to light.

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## EFFECT OF POTATO EXTRACTS ON GROWTH OF PHYCOMYCES

WILLIAM J. ROBBINS AND KARL C. HAMNER<sup>1</sup>

(WITH THREE FIGURES)

In previous papers ROBBINS (7, 8) concluded that one or more growth substances in addition to thiamin are concerned in the development of *Phycomyces blakesleeanus*. This substance, referred to as factor Z, is produced by *Phycomyces* (9) but not in amounts adequate for its maximum development, especially at or near 25° C.

Factor Z is present in various natural products, including the tubers of the white potato, from which it can be extracted with water or dilute alcohol. In response to its presence, the spore germination, early mycelial growth, and gametic reproduction of *Phycomyces* are increased in a medium of mineral salts, sugar, asparagine, and thiamin. From various observations it seemed probable that factor Z is multiple and does not consist of a single substance. In the present paper evidence on the multiple nature of factor Z is reported. There appear to be at least two substances composing it. If an aqueous solution containing factor Z is shaken with charcoal, one fraction is adsorbed by the charcoal and another is not; it remains in the aqueous filtrate following filtration of the mixture.

### Material and methods

Two potato extracts were prepared.<sup>2</sup> Extract P<sub>2</sub> was obtained as follows. Six kg. fresh potatoes were ground in a food chopper, after removing the peel. The pulp was covered with 2.5 liters of 60 per cent methanol and allowed to stand for 18 hours at room temperature. The extract was strained through cheesecloth, centrifuged, and the supernatant liquid heated to boiling. The coagulum which formed was removed by centrifuging, the liquid evaporated to 150 ml. and

<sup>1</sup> Assistance in this work was furnished by the personnel of Works Projects Administration Official Project 65-1-97-23 W.P. 5. The work was aided in part by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago. Additional cost of publication was borne by the authors.

<sup>2</sup> The assistance of Frederick Kavanagh aided in preparing these extracts.

extracted twice with n-butanol. The aqueous residue was evaporated and made up to 125 ml. It was a dark brown solution and contained 61.8 per cent solids. One ml. represented the extract of 48 gm. of potatoes.

Extract P<sub>17</sub> was prepared as follows. Thirty kg. of peeled and sliced potatoes were covered with 50 per cent methanol for 18 hours. The liquid was decanted, the starch allowed to settle, and removed. The alcoholic liquid was then evaporated to about 500 ml. and allowed to crystallize in the refrigerator. The crystals were removed, dissolved in water, and recrystallized. The two supernatant liquids, the one remaining after the first crystallization and the one remaining after the second, were combined and treated with an equal volume of methanol and further precipitation completed in the cold. The precipitate was removed, dissolved in water, and reprecipitated. The liquid fractions remaining after the two precipitations were made up to 70 per cent methanol. The precipitate which formed was removed, dissolved in water, and reprecipitated. The several precipitates were discarded, but all the liquid fractions were combined, evaporated nearly to dryness, and then made up to 100 ml. with water. This dark brown liquid contained 67.6 per cent solids, and 1 ml. represented the extract from 300 gm. potatoes.

In culturing the fungus, each liter of the basal solution (solution I) used in these experiments contained 50 gm. dextrose, 2 gm. asparagine, 1.5 gm. KH<sub>2</sub>PO<sub>4</sub>, 0.5 gm. MgSO<sub>4</sub> · 7 H<sub>2</sub>O, 0.5 mg. thiamin, and the following trace elements: 0.005 ppm B, 0.02 ppm Cu, 0.1 ppm Fe, 0.01 ppm Ga, 0.01 ppm Mn, 0.01 ppm Mo, and 0.09 ppm Zn. All chemicals were of chemically pure grade; the dextrose was Corn Products Company chemically pure; the thiamin, Merck's synthetic; the asparagine was purified by precipitation from alcohol. In testing the effect of various extracts on spore germination, growth, and zygote formation, various amounts of the extracts were added to aliquots of the basal solution (solution I) and the response of the fungus to these additions of the extract was followed. All solutions were sterilized at 110° C. just prior to inoculation.

For spore germination tests, agar was added to the liquid media to make a 1 per cent mixture which solidified when poured into petri dishes. Drops of a spore suspension of *Phycomyces* were placed on

the agar and the percentage of germinated spores counted 12-15 hours after incubation at 23°-25° C.

For growth studies, 25 ml. of solution were used in 125 ml. Erlenmeyer flasks. A drop of a spore suspension was used as inoculum and the cultures were incubated approximately 72 hours at 23°-25° C. The mycelium was filtered into Gooch crucibles, washed, dried at 100° C., and weighed. The results are recorded in milligrams of dry mycelium.

For some studies of gametic reproduction the solutions were poured into petri dishes, 12.5 ml. per dish, and the liquid inoculated with a drop of a spore suspension of both the plus and minus strains. The cultures were incubated at 23°-25° C. and the production of progametes and zygotes noted. In other studies a drop of the spore suspensions of each of the plus and minus strains was placed at opposite ends of a diameter on an agar plate and the production of progametes and zygotes noted after incubation for 8 days.

### Experimentation

EFFECT OF POTATO EXTRACT.—Various quantities of an extract of potato (P<sub>2</sub>) were added to aliquots of solution I containing 1 per cent Difco agar. Twenty-five ml. quantities of each resulting solution were poured into sterile petri dishes. Drops of a spore suspension of the plus strain of *Phycomyces* were placed on the medium and the plates incubated for 15 hours at 23°-25° C. The experiment was performed in duplicate and the results were as follows:

DRY MATTER OF POTATO EXTRACT P <sub>2</sub> PER PLATE (MG.)	PERCENTAGE GERMINATION	DRY MATTER OF POTATO EXTRACT P <sub>2</sub> PER PLATE (MG.)	PERCENTAGE GERMINATION
0.....	0.36	31.....	38.5
1.5.....	0.7	78.....	72.5
7.7.....	2.2	155.....	90.0
15.0.....	4.0		

It is evident that this extract had a marked effect in increasing the spore germination of *Phycomyces* at 23°-25° C. in an agar medium containing mineral salts, sugar, asparagine, and thiamin. Potato extract also had a distinct effect in increasing the rate of growth of the mycelium of *Phycomyces*. To 25 ml. quantities of solution I various quantities of extract P<sub>2</sub> were added and each resulting solution inoculated with a drop of a spore suspension of the plus strain

of *Phycomyces*. After 3 days of incubation at 23°–25° C. the dry weight of the mycelium was determined with the following results (also shown in figure 1):

P <sub>2</sub> ADDED TO EACH FLASK (ML.)	DRY MYCELIUM PRODUCED (MG.)	P <sub>2</sub> ADDED TO EACH FLASK (ML.)	DRY MYCELIUM PRODUCED (MG.)
0.000.....	1.8	0.05.....	88.9
0.001.....	2.3	0.1.....	113.8
0.005.....	8.9	0.2.....	129.2
0.01.....	27.8	0.5.....	87.4
0.02.....	54.1		

One ml. of extract P<sub>2</sub> contained 618 mg. dry matter.

In another experiment designed to test its effect on the gametic reproduction of *Phycomyces*, various quantities of P<sub>2</sub> were added

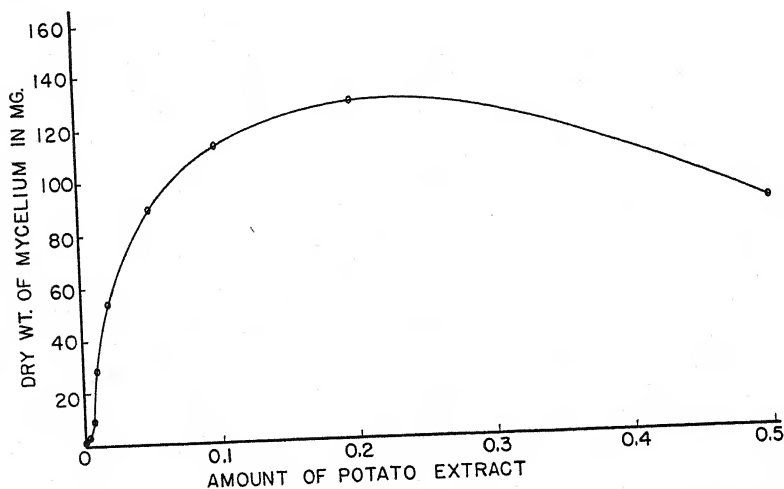


FIG. 1.—Effect of potato extract (amount used expressed in ml. of extract per plate) upon early growth of *Phycomyces* at 23°–25° C. in a solution of mineral salts, sugar, asparagine, and thiamin.

to solution I containing 1 per cent Difco agar purified by extraction with 5 per cent aqueous pyridine (7). Twenty-five ml. of these media were poured into petri dishes and inoculated at opposite ends of a diameter with plus and minus strains of *Phycomyces*. The experiment was set up in quadruplicate. The cultures were incubated at 23°–25° C. for 8 days and then observations on gametic reproduction were made. The results are shown in table 1 and figure 2.

TABLE 1  
EFFECT OF POTATO EXTRACT P<sub>2</sub> ON PRODUCTION OF  
PROGAMETES AND ZYGOTES BY PHYCOMYCES

EXTRACT P <sub>2</sub> ADDED PER PLATE (ML.)	DRY MATTER ADDED PER PLATE (MG.)	LENGTH OF LINE PRO- GAMETES IN CM. ON PLATES 9 CM. IN DIAMETER	No. ZYGOTES PER PLATE
0.25.....	155	9.0	143
0.20.....	124	9.0	175
0.15.....	93	9.0	258
0.10.....	62	9.0	236
0.075.....	46	9.0	87
0.050.....	31	9.0	50
0.040.....	25	9.0	38
0.030.....	18.6	9.0	32
0.020.....	12.4	3.2	1
0.015.....	9.3	1.7	None
0.010.....	6.2	0.7	None
0.005.....	3.1	None	None
0.0025.....	1.5	None	None
0.00125.....	0.7	None	None
0.0005.....	0.4	None	None
None.....	None	None	None

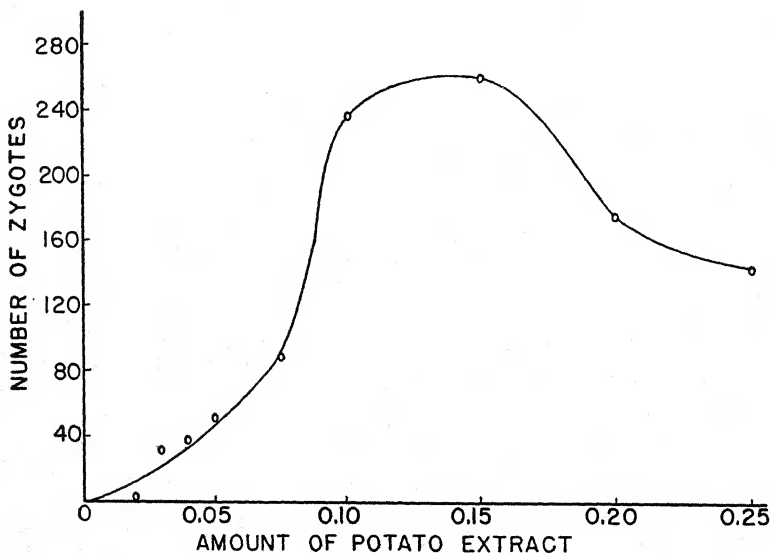


FIG. 2.—Effect of potato extract (amount used expressed in ml. of extract per plate) upon gametic reproduction of *Phycomyces* on agar medium containing mineral salts, sugar, asparagine, and thiamin.

Growth was least vigorous in the plates to which no potato extract was added. In those plates where no progametes occurred the mycelium covered the surface of the medium and abundant sporangio-phores and sporangia were produced.

FRACTIONATION OF POTATO EXTRACT.—In the course of observations on the effect of various extracts from several sources, including those prepared from potato, upon the development of *Phycomyces*, there were indications that some extracts resulted in increased growth but not gametic reproduction, while others appeared to result in an increase of both growth and gametic reproduction. It seemed desirable, therefore, to fractionate the potato extract and determine if possible whether more than one factor present in potato extract was concerned in its resultant effects.

A quantity of an extract of potato ( $P_{17}$ ) was treated with ethanol to form a 93 per cent alcoholic solution. The precipitate which formed was removed, dissolved in water and treated in slightly acid solution with norit A, and filtered. The filtrate was evaporated to dryness in partial vacuum and taken up with  $H_2O$ . This solution was designated  $P_{17}B$ . The norit, which remained on the filter, was shaken with 5 per cent aqueous pyridine and the resulting solution freed from pyridine by evaporation to dryness in partial vacuum. The residue was taken up with  $H_2O$  and designated  $P_{17}A$ . The original filtrate from the treatment of the  $P_{17}$  extract with 93 per cent alcohol was evaporated to dryness in partial vacuum, taken up with water and treated with norit in slightly acid solution. This mixture was then filtered, and the filtrate labeled  $P_{17}D$ . The norit, which remained on the filter, was then treated with 5 per cent aqueous pyridine, filtered, and the resulting solution freed from pyridine by evaporation and designated  $P_{17}C$ . These four fractions were diluted with distilled water so that 1 ml. was equivalent to the extract from 50 gm. of fresh potato. Each of the four extracts was tested for activity as it affected growth of the fungus in a manner similar to that already described for potato extract  $P_2$ .

For brevity, these solutions are designated respectively as A, B, C, and D. Extracts A, B, and C showed slight activity; extract D was about two-thirds as active in promoting development of the fungus as the original potato extract  $P_{17}$ . On the basis of a comparison of the dry matter in the original extract with the total of that in the four

fractions, it was found that some 15 per cent of the original dry matter could not be accounted for.

On the assumption that this material had been adsorbed by the norit and not freed by the pyridine treatment, the norit from which fraction  $P_{17}A$  was obtained and that from which  $P_{17}C$  was obtained were each treated with ammoniacal acetone (60 per cent acetone and 2.5 per cent ammonia). Each norit and acetone mixture was shaken for several hours and filtered. The norit was extracted in this manner with ammoniacal acetone three times and the acetone extracts from each individual lot of norit combined, evaporated to dryness in partial vacuum, and the residues taken up in enough water so that 1 ml. of solution was equivalent to 50 gm. of fresh potato. The two solutions thus obtained, one from the norit of fraction  $P_{17}A$  and one from the norit of fraction  $P_{17}C$ , are designated Aa and Ca, respectively. Aa had very little effect in promoting development of *Phycomyces* and Ca was decidedly more active than C, although considerably less so than the original extract  $P_{17}$  or fraction D. It appeared from these results that two active fractions had been secured from potato: one (Ca) was adsorbed on charcoal from a weakly acid solution and freed from the charcoal by ammoniacal acetone; the other (fraction D) was in the charcoal filtrate. Fraction D was again treated with norit until all color was removed and the colorless filtrate designated fraction Dr. Both fractions, Ca and Dr, were adjusted with distilled water so that 1 ml. was equivalent to the extract from 50 gm. potato. Fraction Ca contained 0.51 per cent solids and Dr 5.71 per cent.

EFFECT OF FRACTIONS CA AND DR ON GROWTH AND GAMETIC REPRODUCTION.—Preliminary experiments indicated that Ca affected mycelial growth but had little effect upon the formation of zygotes, while Dr had a greater effect on growth and also increased gametic reproduction.

The effect of the extracts on growth and gametic reproduction was determined by the addition of various quantities of Ca and Dr, both separately and in combination, to solution I. For determining the effect on gametic reproduction, two methods were employed. In one, duplicate petri dishes each containing 12.5 ml. of solution I and inoculated with a drop of the plus and the minus strains were used. In the other duplicate petri dishes each containing 25 ml. of solution I to which had been added 1 per cent agar which had been purified

by extraction with pyridine were employed. The agar plates were inoculated with the plus and minus strains at opposite ends of a diameter. In studying the effects of the extracts on growth, the dry weight of the mycelium was determined after 3 days' incubation by the method described in the use of the extract P<sub>2</sub>. All cultures were incubated at 23°–25° C.

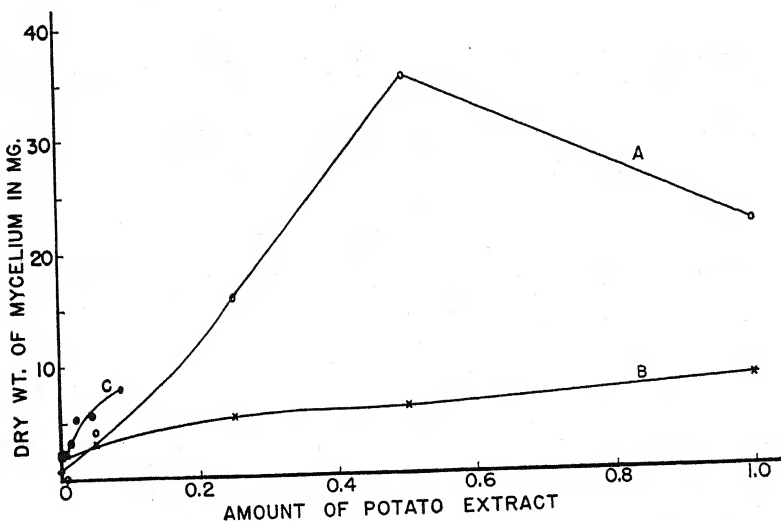


FIG. 3.—Effect of fractions of potato extract on early growth of *Phycomyces*: A, charcoal filtrate; B, charcoal adsorbate; C, effectiveness of charcoal adsorbate expressed in terms of dry matter added, the units on the abscissa conforming with equivalent amounts of dry matter of charcoal filtrate as represented in A.

Fractions Ca and Dr both brought about an increase in the rate of growth of *Phycomyces* when either was added to solution I (fig. 3). The Dr fraction produced more effect per ml. of extract used, but since this fraction contained more dry matter per ml. than Ca, both fractions were about equally effective on the basis of dry matter added. An effect was noted with the addition of as little as 25 gamma of dry matter of fraction Ca.

From a comparison of the effects of the two fractions, when used alone, with that of their effects when used in combination (table 2), it would appear that each contained some promotive factor or factors which was more effective in the presence of the other than when used

alone. For example, the presence of 1 ml. of Dr resulted in 22.1 mg. of mycelium and the presence of 0.005 ml. of Ca in 2.1 mg. of growth as compared with 1.4 mg. produced when neither was present. The additions of both these (that is, 1 ml. of Dr and 0.005 ml. of Ca) to the same flask resulted in 81.1 mg. of growth. Numerous other such examples may be found in table 2. Thus, addition of small amounts of both fractions to the same culture resulted in far greater mycelial growth than might be expected on the basis of a consideration of the individual effects produced by either fraction alone.

TABLE 2  
COMBINED AND INDIVIDUAL EFFECTS OF FRACTIONS CA AND DR  
OF POTATO EXTRACT ON GROWTH OF PHYCOMYCES IN BASAL  
SOLUTION CONTAINING THIAMIN. MG. DRY WEIGHT OF MY-  
CELIUM

EXTRACT CA PER 25 ML. MEDIUM (ML.)	ML. OF FRACTION DR PER 25 ML. MEDIUM					
	1.0	0.5	0.25	0.05	0.005	0
1.0 . . . . .	35.7	47.1	30.8	10.6	7.9	8.2
0.5 . . . . .	68.1	48.3	33.7	11.8	5.5	5.9
0.25 . . . . .	59.5	84.3	42.5	10.9	4.3	5.2
0.05 . . . . .	65.2	81.5	24.2	9.5	3.2	2.9
0.005 . . . . .	81.1	77.9	22.7	11.8	1.8	2.1
0.000 . . . . .	22.1	35.8	16.0	3.9	0.1	1.4

The effect of the combined extracts on gametic reproduction also was greater than that of either alone. Few progametes developed in the plates containing the liquid medium to which the Ca fraction was added and none on the check plates (table 3). In those to which 1 ml. of the Dr fraction was added, the surface of the mycelium was nearly covered with progametes. The quantity of progametes, however, was increased by the addition of both fractions. In those containing 0.5 ml. of the Dr fraction scattered groups of progametes appeared, and the further addition of 0.25 ml. and 0.5 ml. of fraction Ca resulted in marked increase in their production. The character of the mycelial growth in the plates with fraction Ca differed from that in those containing the Dr fraction. In the latter more colonies developed with a dry surface; in the former the colonies were fewer and larger and remained submerged for a longer period.

On the agar plates also the production of progametes was greater

where the two fractions were combined than where they were used singly. However, more progametes were formed on the agar with the larger quantities of fraction Ca than in the liquid plates, suggesting that the agar may have contained some of the Dr factor, not removed in the process of purification by pyridine.

TABLE 3  
EFFECT OF TWO FRACTIONS OF POTATO EXTRACT ON FORMATION OF  
PROGAMETES IN LIQUID MEDIA. RELATIVE PRODUCTION  
OF PROGAMETES PER PLATE

ML. OF FRACTION Ca PER 25 ML. MEDIUM	ML. OF FRACTION Dr PER 25 ML. MEDIUM					
	1.0	0.5	0.25	0.05	0.005	0
1.0.....	Heavy	Heavy	Few	Few	Very few	Very few
0.5.....	Heavy	Heavy	Few	Few	Few	Few
0.25.....	Heavy	Heavy	Few	Few	Few	Very few
0.05.....	Heavy	Scattered groups	Few	Few	Few	Few
0.005.....	Heavy	Scattered groups	Few	.....	.....	None
0.....	Heavy	Scattered groups	Few	Few	Very few	None

TABLE 4  
EFFECT OF TWO FRACTIONS OF POTATO EXTRACT ON  
PERCENTAGE OF SPORE GERMINATION BY  
PHYCOMYCES

ML. OF FRACTION Dr PER 25 ML. OF MEDIUM	ML. OF FRACTION Ca PER 25 ML. OF MEDIUM			
	1.0	0.5	0.25	0
1.0.....	62.4	71.0	64.1	31.8
0.5.....	50.6	47.9	42.2	15.2
0.25.....	.....	.....	.....	26.0
0.....	10.9	6.0	0.5	1.2

The two fractions when used together evidenced more than an additive effect upon germination of the spores of *Phycomyces*. In this instance Ca and Dr extracts were freshly prepared. Drops of a spore suspension were placed on solution I containing 1 per cent Difco agar to which various quantities of the two extracts were added (table 4). The plates were incubated at 23°-25° C. and the percent-

age of germination determined after 12 hours of incubation. The plates containing the fraction Dr exhibited a higher percentage of germination than those containing equivalent volumes of Ca. When the two fractions were combined the effect was considerably greater than would be expected on an additive basis.<sup>3</sup>

INFLUENCE OF ANTI GRAY-HAIR FACTOR.—Through the courtesy of Dr. AGNES MORGAN, of the University of California, a concentrate of the anti gray-hair factor prepared from yeast was received. Added to solution I this concentrate had a marked effect in stimulating the early growth of *Phycomyces*. One mg. of the concentrate in 25 ml. of

TABLE 5  
EFFECT OF ANTI GRAY-HAIR FACTOR CONCENTRATE AND  
FRACTION DR OF POTATO EXTRACT ON GROWTH OF  
PHYCOMYCES. MG. DRY WEIGHT OF MYCELIUM

AMOUNT OF ANTI GRAY-HAIR FACTOR IN GM. SOLIDS ADDED TO 25 ML. OF MEDIUM	ML. OF FRACTION DR PER 25 ML. MEDIUM		
	1.0	0.25	0
0.04.....	43.3	29.1	21.7
0.02.....	140.9	111.8	32.2
0.004.....	140.3	113.3	42.9
0.001.....	129.7	58.4	3.9
0.....	22.1	16.0	2.3

solution I was sufficient to produce a definite increase in growth; larger quantities produced greater increases (table 5), the increases becoming relatively smaller with the largest amounts used. Although the effect of the anti gray-hair factor concentrate increased the growth of *Phycomyces* in the presence of thiamin, it had relatively little effect upon the gametic reproduction in liquid plates. In fact the general effect of the concentrate upon development of *Phycomyces* resembled that of the charcoal adsorbate prepared from potato tubers. The similarity between the effects of the anti gray-hair factor concentrate and the Ca fraction was confirmed by studying the effect of combinations of the concentrate with each of the two fractions of the potato extract. When various amounts of frac-

<sup>3</sup> The assistance of Leon Grodsinsky in performing this experiment is gratefully acknowledged.

tion Ca—ranging from 0.005 to 1 ml. per flask—were added to solution I containing the anti gray factor, no marked increase in growth of *Phycomyces* occurred. On the other hand, combinations of the concentrate with the fraction Dr gave high yields, considerably larger than could be accounted for on an additive basis (table 5). Furthermore, the anti gray-hair factor concentrate with the fraction Dr markedly favored gametic reproduction, some combinations resulting in thousands of zygotes with black appendages. The production of progametes with solutions supplemented with the anti gray concentrate alone was relatively scant. Although additions of sufficient extract Dr alone caused profuse production of progametes, no zygotes with appendages were formed.

ATTEMPTS AT IDENTIFICATION OF EFFECTIVE FRACTIONS OF EXTRACTS.—It was thought that the factor Dr might be m-inositol, which has been demonstrated by EASTCOTT (2) to be a part of the bios complex and found by BUSTON and PRAMANIK (1) to be one of the two growth substances required by *Ashbya gossypii*; however, the addition of 20 mg. per liter of inositol to solution I containing 0.005–0.25 ml. of extract Ca per culture had no effect upon the development of *Phycomyces*. Inositol alone added to solution I was ineffective.

It was reported previously (8) that vitamin B<sub>2</sub>, vitamin B<sub>6</sub>, and nicotinic acid did not increase growth of *Phycomyces* in the presence of thiamin. A combination of these three growth substances together with adenine sulphate and  $\beta$  alanine used in the presence of thiamin and extract Ca was ineffective. The following mixture was added to each flask containing 25 ml. of solution I:

	Mg.
Vitamin B <sub>2</sub> (lactoflavin).....	0.0038
Vitamin B <sub>6</sub> .....	0.005
Nicotinic acid.....	0.000005
$\beta$ alanine.....	0.005
Adenine sulphate.....	0.005

The amounts of m-inositol and other growth substances listed were arbitrarily selected. Other quantities might act differently, although we have no reason at present to anticipate such an eventuality.

### Discussion

Interpretation of the effects of extracts of natural products upon development of an organism depends in part upon the conditions under which the extracts are used. If the basic medium already contains luxur amounts of the essential mineral salts, as well as available carbon and nitrogen compounds, any benefit from the extracts cannot be ascribed to an addition to the supply of these classes of materials. Of course, not all mineral salts and not all compounds of carbon and nitrogen are equally available. Iron, for example, may be present in large amounts and the organism still suffer from a deficiency because the form of the iron is relatively unavailable. The quality of nutrients and major foods as well as their quantity must therefore be borne in mind. Furthermore, the total amount of growth must be considered. None of the nutrients or foods may limit growth in a given medium if the period of growth is short and the total tissue which develops is small, yet with longer time and greater growth one or more of these materials may become limiting factors.

We believe that the medium used in our experiments contains mineral nutrients, foods, and thiamin in amounts exceeding the needs of the organism for the period of the experiments. This is obviously true for the experiments on spore germination where the demand for nutrients and foods is distinctly limited. A similar condition exists for a 3-day growth period during which but a few milligrams of mycelium are produced in the basic solution. Furthermore, extremely small amounts of the extract (25 gamma of Ca) were found to be effective, which also would suggest the action of a growth substance. Considerations of this character, as well as others previously discussed (7, 8, 9), have led to the assumption that an unidentified growth substance or substances, factor Z, is present in potato extracts and is responsible for their beneficial action.

If this assumption is correct, then it would seem to follow that factor Z consists of at least two growth substances, one which can be adsorbed on charcoal and one which after filtration would be present in the filtrate. It is proposed to refer to the former as factor  $Z_1$  and to the latter as factor  $Z_2$ . Unless the existence of two factors is assumed, it is difficult to explain why the charcoal adsorbate and the charcoal filtrate when used alone should be so much less effective on spore germination, early mycelial growth, and gametic reproduc-

tion than when used in combination. If either or both extracts were toxic it might be assumed that the phenomenon of antagonism was involved, but each extract is beneficial, which would appear to eliminate that possibility.

The  $Z_1$  factor appeared to be concerned primarily in growth and had little effect upon gametic reproduction when used alone with thiamin. On the other hand, the  $Z_2$  factor affected favorably both growth and gametic reproduction.

From various observations made during the course of our experiments a suitable proportion of the two factors seemed desirable for optimum results. Too much of fraction Ca (containing the  $Z_1$  factor) in proportion to fraction Dr (containing the  $Z_2$  factor) appeared, for example, less satisfactory for gametic reproduction than mixtures with smaller amounts of fraction Ca. Of course these conclusions may be modified when purer preparations are available.

It is perhaps premature to discuss the identities of the two factors, but some possibilities may be mentioned.

Factor  $Z_1$  has some of the characteristics of biotin. Both are adsorbed by charcoal, from which they may be freed by ammoniacal acetone, and both are thermostable. Biotin has been reported by KÖGL and FRIES (3) to be ineffective for *Phycomyces*, in the presence of thiamin. That would not necessarily eliminate the possibility that factor  $Z_1$  is biotin. Our preparations are impure and the Ca fraction might contain traces of the  $Z_2$  factor, which would account for the beneficial effect of the Ca fractions when used alone. If  $Z_1$  is biotin, it might be assumed that development of *Phycomyces* in a thiamin solution under the conditions we have used is limited by its ability to synthesize factor  $Z_2$ . When that factor is supplied, its development is limited by its ability to synthesize factor  $Z_1$ . Under such a hypothesis biotin would be ineffective when used with thiamin and effective when used together with thiamin and factor  $Z_2$ . Further investigation of the possible identity of factor  $Z_1$  and biotin is under way.

The experiments with the anti gray factor concentrate show that it contains considerable  $Z_1$  factor. It is uncertain, however, whether the anti gray factor and the  $Z_1$  factor are identical. The former is thermostable, resisting autoclaving at 10–15 pounds' pressure for 15 minutes and some hours of boiling temperature; the latter according

to LUNDE and KRINGSTAD (5) is destroyed by 3 hours' boiling in vacuo. On the other hand, MORGAN (6) reports that a yeast eluate heated under a reflux in strongly alkaline solution still retains its activity. The anti gray factor concentrate probably contains several growth substances; we have found, for example, that there is considerable biotin present—as determined by the growth of *Ashbya gossypii* (10).

The possibility that the  $Z_1$  factor might be pantothenic acid was considered also, although pantothenic acid (11) has been reported ineffective for *Phycomyces*. But this acid would appear to be more easily destroyed by heat than is the  $Z_1$  factor.

Consideration should be given also to the rat growth factor reported by LEPKOVSKY, JUKES, and KRAUSE (4). This factor is adsorbed by charcoal and is resistant to heating, as reported by LUNDE and KRINGSTAD (5).

The  $Z_2$  factor does not appear to be m-inositol or vitamin  $B_2$ , vitamin  $B_6$ , nicotinic acid,  $\beta$  alanine, or adenine.

The possibility that there are more than two factors concerned cannot be neglected. At this stage, however, it would not be advisable to assume more than two.

### Summary

1. Evidence has been presented which confirms previous reports that spore germination, early growth, and gametic reproduction in *Phycomyces* may be stimulated in cultures to which a potato extract has been added. Increasing amounts of potato extract in the culture medium up to a certain amount may increase growth and reproduction; quantities beyond these amounts bring about decrease in growth and zygote formation.

2. Indications are that the potato extract used contains at least two factors which influence development of *Phycomyces*. One factor can be adsorbed on charcoal and is freed from it in the presence of ammoniacal acetone. This factor is designated at present  $Z_1$ . The other factor is not adsorbed to any great extent on charcoal and following filtration is present in the filtrate. This factor is designated  $Z_2$ .

3. Either of the factors, when used in the relatively impure state described here, produced some stimulative effect upon development, but a combination of the two stimulated development far more than either used alone.

4. A concentrate of the anti gray factor obtained from Dr. AGNES MORGAN stimulated growth of *Phycomyces* when used alone in solution I but was much more effective when used in combination with the fraction containing the  $Z_2$  factor. This may indicate that the anti gray concentrate is especially rich in the  $Z_1$  factor.

5. The  $Z_1$  factor may be identical with biotin. The properties of the  $Z_2$  factor have not as yet been correlated with those of any other known growth factor or vitamin.

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# AVENA COLEOPTILE ASSAY OF ETHER EXTRACTS OF APHIDS AND THEIR HOSTS<sup>1</sup>

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 513

GEORGE K. K. LINK, VIRGINIA EGGERS, AND JAMES E. MOULTON

## Introduction

This investigation was carried out to determine, by the standard decapitated *Avena* coleoptile test (12), whether ether extracts of aphids are active in this test; and if so, to compare them with ether extracts of the host organ as to kind and magnitude of effect. The study proceeded from the hypothesis that if growth affecting or effecting substances, *i.e.*, auxones (3, 4, 5) play the roles in growth indicated by current theory and experiment, then the growth disturbances noted in many plants parasitized by aphids should be correlated with disturbed auxone relations (dysauxony), and the aphids should play causal roles in these disturbances by affecting the kind, quantity, behavior, and other relations of the relevant auxones, including auxins and auxin inhibitors, if these be involved. Theoretically, the aphid could disturb these relations in part (a) by secreting or excreting auxones on to or into the host, and/or (b) by withdrawing auxones from the host. Addition, withdrawal, or both, of auxones of the auxin, auxin inhibitor, vitamin, or wound types could disturb the auxone balances characteristic of normal healthy tissues and organs (euauxony).

To date we have tested the extracts of three aphids and their hosts: (1) *Hyalopterus arundinis* on *Phragmites communis*; (2) *Aphis maidis* on *Zea mays*; and (3) *Brevicoryne brassicae* on *Brassica oleracea*. We started with *Hyalopterus arundinis*, even though it does not produce serious local growth disturbances in *Phragmites* leaves, because the large quantities (100 gm.) which then we considered necessary could be collected rather readily. *Aphis maidis* was used because it was available as another grass parasite. *Brevicoryne brassicae* was used because it often incites serious local growth disturb-

<sup>1</sup> This work was aided in part by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

ances in cabbage and because the aphid and host can readily be kept in a thrifty condition in the greenhouse during the Chicago winter. Because of the latter fact, most of our tests were made with the cabbage aphid and cabbage leaf.

### Investigation

#### GENERAL METHODS

Removal of aphids into a glass container by means of a brush of proper stiffness was found the most feasible way to collect them with minimum injury. At the time of collection we also took aphid-free leaf samples of the same rank as the leaves from which aphids were collected.

After weighing, the aphid and leaf sample each was placed in peroxide-free ether (50 cc. per gram fresh material) and extracted in the dark at 0°C. for 22 hours. The ether was then separated from the sample, and from water, evaporated to dryness over a steam-bath, and the residue stored overnight at 0°C. in the dark (9). On the next day the residue, here designated extract, was taken up in 2 cc. of melted 1.5 per cent agar, and from this mixture dilutions were prepared by addition of agar so that each new dilution was one-half as concentrated as the previous one. In several experiments, on the day of testing the ether was evaporated to 1-2 cc. and then dropped on to 2 cc. of hot 1.5 per cent agar, from which the initial dilution was made.

The agar dilutions were poured into an  $8 \times 10 \times 1.5$  mm. mold. After hardening, the agar cast was cut into twelve equal blocks. These were applied to twice decapitated *Avena* coleoptiles. In each dilution (concentration) tested at least twelve coleoptiles were used; in some tests twenty-four were used. Controls of indoleacetic acid in concentrations of 10, 20, 30, and 40 $\gamma$  were run on each day of test, twelve or more coleoptiles per concentration. The curvatures were recorded after 90 minutes. For comparison, the curvature of the 40 $\gamma$  indoleacetic acid solution is recorded for each day for testing, although this concentration lies slightly above the proportionality range.

Several preliminary tests were made to determine the kinds of constituents in the extracts. To this end we used VAN OVERBEEK and BONNER's adaptation of the KÖGL, HAAGEN-SMIT and ERXLE-

BEN procedure (10) to determine the effect of refluxing with water, 5 per cent HCl, and 0.5 N NaOH upon the activity of different lots of the extracts of *Hyalopterus arundinis* and *Brevicoryne brassicae* and of their hosts. After cooling, each lot was adjusted to slightly acid, and then extracted with ether, the ether separated and evaporated, and the residue taken up in agar and tested.

EXTRACTS OF HYALOPTERUS ARUNDINIS AND  
PHRAGMITES COMMUNIS

The aphids were covered with honey dew at the time of collection. The plants were vigorous and collections were made on hot July days. Table 1 gives the results of tests with three concentrations of the ether extract of a 1-gm. sample of the aphid.

TABLE 1  
ACTIVITY OF ETHER EXTRACT OF HYALOPTERUS ARUNDINIS  
EXPRESSED IN AVERAGE CURVATURE PER 12 AVENA  
COLEOPTILES (DEGREES)

TEST	MATERIAL	AMOUNT EXTRACTED (GM.)	AMOUNT TESTED (GM.)*		
			0.5	0.25	0.125
20.....	Aphid	1.0	9.8	7.1	2.6
Indoleacetic acid 40γ = 19.7°					

\* No. of grams of fresh material extracted represented by its portion of extract in 1 cc. of 1.5% agar.

The extracts of *Hyalopterus arundinis* and of *Phragmites* leaf are active in the coleoptile test (tables 1 and 2). There is a suggestion of proportionality between concentration and activity of the extracts. Lower concentrations of the aphid extract gave better proportionalities than higher ones. Since the lower concentrations of aphid extract and the higher concentrations of leaf extract did not seem to be supramaximal, the greater activity noted for the aphid extract may signify that this differs from the leaf extract as to amount and kind of its effective substances. The extract of the aphid, and possibly of the leaf, each contains a fraction which is active after acid, and another which is active after alkali, hydrolysis (4).

## EXTRACTS OF APHIS MAIDIS AND ZEA MAYS

The plants used for collection of aphids, leaves, and tassels were grown in the greenhouse and had shed their pollen at time of collection. The aphids were rich with honey dew.

TABLE 2

ACTIVITY OF NON-REFLUXED AND REFLUXED ETHER EXTRACTS OF HYALOPTERUS ARUNDINIS AND OF PHRAGMITES LEAF, EXPRESSED IN AVERAGE CURVATURE PER 12 AVENA COLEOPTILES (DEGREES)

TEST	TREATMENT	MATE- RIAL	AMOUNT EXTRACT- ED (GM.)	AMOUNT TESTED (GM.)*			
				5.0	2.5	1.25	0.625
21 } 22 }	Not refluxed	{Aphid Leaf	10	16.0	18.0	10.0	11
			10	4.3	1.9	1.5	.....
21 } 22 }	H <sub>2</sub> O refluxed	{Aphid Leaf	10	15.0	18.0	13.0	.....
			10	3.2	0.2	2.6	.....
21 } 22 }	5% HCl refluxed	{Aphid Leaf	10	6.3	15.0	13.0	.....
			10	2.2	2.3	1.5	.....
21 } 22 }	0.5 N NaOH re- fluxed	{Aphid Leaf	10	9.4	3.6	5.5	.....
			10	3.0	1.5	1.0	.....
21	Indoleacetic acid 40γ=18°						
22	Indoleacetic acid 40γ=19.7						

\* No. of grams of fresh material extracted represented by its portion of extract in 1 cc. of 1.5% agar.

TABLE 3

ACTIVITY OF ETHER EXTRACTS OF APHIS MAIDIS AND OF LEAVES AND TASSELS OF ZEA MAYS, EXPRESSED IN AVERAGE CURVATURE PER 12 AVENA COLEOPTILES (DEGREES)

TEST	MATERIAL	AMOUNT EXTRACTED (GM.)	AMOUNT TESTED (GM.)*					
			10.0	5.0	2.5	1.25	0.125	0.062
27.....	{ Aphid from leaf	5	.....	.....	.....	7.7	9.0	4.5
	Leaf	20	40.0	33.5	26.0	.....	.....	.....
	{ Aphid from tassel	5	.....	.....	.....	9.2	11.7	15.0
	Tassel	20	14.2	12.7	14.0	.....	.....	.....
Indoleacetic acid $40\gamma = 24.3^\circ$								

\* No. of grams of fresh material extracted represented by its portion of extract in 1 cc. of 1.5% agar.

The extracts of aphids collected from corn leaves and tassels, as well as the extracts of those organs, were active in the coleoptile

test (table 3). There was no linear proportionality between activity and concentration. While the extract of aphids from the tassel was more active than the extract of the leaf aphid, the extract of the leaf was more potent than the tassel extract for the same concentrations, possibly indicating that these extracts are not identical as to kind and quantity of effective substances. The leaf extract produced pronounced curvatures ( $40^{\circ}$ - $26^{\circ}$ ), whereas the highest curvature noted on the day of testing for indoleacetic acid was  $24.3^{\circ}$  for a solution containing 40 $\gamma$  per liter. This may indicate that the leaf extract contains a substance or mixture of substances which does not behave like pure indoleacetic acid in the coleoptile test.

EXTRACTS OF BREVICORYNE BRASSICAE AND OF LEAVES  
OF BRASSICA OLERACEA

A great many tests were made with these materials, only a few of which are reported here (tables 4, 5, and 6). For tests 33, 34, and 36 (table 4) the aphids were collected from leaves of various sizes, and comparable aphid-free leaves were used as leaf samples. For tests with small samples (tables 5 and 6) only portions of a single leaf were used. Prior to and at the time of collection, the plants were in a warm room. Collections were made on sunny days from potted plants which had 8-10 leaves each. The aphids were very mealy and of various sizes.

The extracts of the cabbage aphid and cabbage leaf were found active. Each contains a fraction which remains active after acid hydrolysis and one which is active after alkali hydrolysis (table 4). The differences in activity of these fractions of the extracts from 0.62-gm. samples may indicate that the effective substances of the extracts of the aphid and of the leaf differ as to kind and quantity.

Many tests were made in an attempt to find concentrations which would give proportionalities between concentration and activity. The results are so variable from one test to another, and the number of tests possible per day is so limited, that we have not yet succeeded. Variability in leaves and aphids may be due to the fact that even when leaves strictly comparable as to rank and age are used, these leaves and the aphids on them are not the same as to auxone content because they have not experienced the same light and other conditions. If leaves of different ages are used the differences are even greater.

TABLE 4

ACTIVITY OF NON-REFLUXED AND REFLUXED ETHER EXTRACTS OF BREVICORYNE BRASSICAE AND OF LEAVES OF BRASSICA OLERACEA, EXPRESSED IN AVERAGE CURVATURE PER 12 AVENA COLEOPTILES (DEGREES)

TEST	TREATMENT	MATERIAL	AMOUNT EXTRACTED (GM.)	AMOUNT TESTED (GM.)*							
				5.0	2.5	0.25	0.02	0.01	0.005	0.0025	0.00125
33	Not refluxed	{Aphid	5	.....	4.8	9.8	10.1	16.9	.....	.....	.....
33		{Leaf	10	1.9	0.5	2.7	.....	.....	.....	.....	.....
34	H <sub>2</sub> O refluxed	{Aphid	5	.....	.....	.....	17.1	13.7	13.0	12	.....
34		{Aphid	5	.....	.....	.....	10.5	17.0	17.0	.....	.....
34	0.5 N NaOH refluxed	{Aphid	5	.....	.....	.....	13.9	9.8	3.0	.....	.....
36		{Leaf	10	.....	0.2	5.0	2.8	.....	.....	.....	.....
34	5% HCl refluxed	{Aphid	5	.....	.....	.....	11.2	13.8	12.0	.....	.....
36		{Leaf	10	.....	0.1	1.4	3.7	.....	.....	.....	.....
33	Indoleacetic acid $40\gamma = 16.2^\circ$										
34	Indoleacetic acid $40\gamma = 20.0$										
36	Indoleacetic acid $40\gamma = 21.0$										

\* No. of grams of fresh material extracted represented by its portion of extract in 1 cc. of 1.5% agar

TABLE 5

ACTIVITY OF ETHER EXTRACTS OF BREVICORYNE BRASSICAE AND OF LEAVES OF BRASSICA OLERACEA, EXPRESSED IN DEGREES CURVATURE OF AVENA COLEOPTILES. DRY RESIDUE KEPT AT 0° C. IN THE DARK AND TESTED AFTER 20 HOURS', 6 DAYS', AND 13 DAYS' STORAGE. AVERAGE CURVATURE PER 12 COLEOPTILES (DEGREES)

DATE (1940)	TEST	MATERIAL	AMOUNT EXTRACTED (GM.)	AMOUNT TESTED (GM.)*							
				0.5	0.25	0.125	0.062	0.031	0.015	0.0075	0.0037
Feb. 2.....	45	{Aphid	0.5	.....	6.6	17.3	14.2	28.4	.....	.....	.....
		{Leaf	1.0	0.4	2.0	2.3	5.8	.....	.....	.....	.....
Feb. 8.....	45a	{Aphid	.....	.....	.....	23.1	.....	17.3	10.6	4.0	.....
		{Leaf	.....	.....	1.0	.....	6.4	21.6	15.4	.....	.....
Feb. 15....	45b	{Aphid	.....	.....	.....	.....	.....	19.8	15.1	11.4	11.9
		{Leaf	.....	.....	.....	.....	4.3	13.3	17.1	16.8	.....
	45	Indoleacetic acid $40\gamma = 30.5^\circ$									
	45a	Indoleacetic acid $40\gamma = 34.4$									
	45b	Indoleacetic acid $40\gamma = 26.1$									

\* No. of grams of fresh material extracted represented by its portion of extract in 1 cc. of 1.5% agar.

In an attempt to avoid this difficulty, different portions of the residue of the same extracts were tested on different days (table 5). The ether extracts of the original samples (0.5 gm. aphids and 1 gm. leaf) each was divided into two equal portions. All were reduced to dryness. One each of these portions was used in test 45, while the other portions were stored as dry residues for 5 days. On the fifth day each of the remaining portions were redissolved in ether, which was then divided into two equal portions so as to give again two

TABLE 6

ACTIVITY OF ETHER EXTRACTS OF BREVICORYNE BRASSICAE AND OF LEAVES OF BRASSICA OLERACEA, EXPRESSED IN AVERAGE CURVATURE PER 24 AVENA COLEOPTILES (DEGREES). CONCENTRATED ETHER EXTRACT DROPPED ON HOT AGAR AND TESTED SAME DAY

TEST	MATERIAL	AMOUNT EXTRACTED (GM.)	AMOUNT USED (GM.)*							
			0.125	0.0625	0.0312	0.0156	0.0078	0.0039	0.0019	0.0009
59....	{Aphid Leaf	0.25	6.4	9.8	18.5	15.7	.....	.....	.....	.....
		0.25	2.6	4.8	6.3	4.4	.....	.....	.....	.....
61....	{Aphid Leaf	0.25	13.4	.....	14.0	.....	3.0	2.0	0.8	0.4
		0.25	4.6	.....	8.9	.....	14.9	8.9	3.8	0.7
59....	Indoleacetic acid $40\gamma = 16.9^\circ$									
61....	Indoleacetic acid $40\gamma = 18.1$ (average per 36 coleoptiles)									

\* No. of grams of fresh material extracted represented by its portion of extract in 1 cc. of 1.5% agar.

portions of aphid extract and two portions of leaf extract. Each was evaporated to dryness and one each of these portions was tested the next day (test 46), while the others were kept for 7 more days and then tested (test 47). Because the values obtained with the first and second tests appeared supramaximal for both aphid and leaf extracts, progressively higher dilutions were used in tests 46 and 47. In addition, some of the dilutions of the preceding tests were used as controls. The extracts were highly active even after 13 days of storage as dry residues. On the thirteenth day, dilutions whose residue contents per cc. of agar represented the extracts of 4-8 mg. of fresh leaf and aphid materials respectively gave pronounced curvatures. Even at these low concentrations no good proportionalities were obtained between activity and concentration.

Because it seemed possible that some of the irregularities in results might be due to the method of dilution used and to changes due to evaporation to dryness, experiments were conducted in which the extracts were not reduced to dryness (table 6). In these tests the ether extracts were evaporated to 1-2 cc. and then dropped on to 2 cc. of hot 1.5 per cent agar. From this mixture 1 cc. was removed; to the remainder, 1 cc. of agar was added to give the second dilution. This was repeated until the desired dilutions (concentrations) had been obtained.

Even though very low concentrations were used in test 59 (0.125-0.0156 gm. of material), no good proportionalities were obtained between concentration and activity. For this reason another collection of leaf and aphids was made from the plants used for test 59. A week intervened between collections. Two of the concentrations used in test 59 were included in test 61 as controls, and in addition four other dilutions were prepared. In order to cover a wide range, the first two concentrations were prepared each by fourfold, and the other four each by twofold, dilutions.

Table 6 indicates that the samples of tests 59 and 61 were not identical as to the active substances under test, showing that comparative quantitative tests are very difficult, if not impossible, under the methods in use at present. The results, unlike those of table 5, also show that the higher dilutions gave better proportionalities between concentration and activity. The best values were obtained with extract equivalents between 1.9 and 30 mg. of fresh material per 1 cc. of agar. The leaf extract was the more active, test 61 indicating that the relatively low values in the higher concentrations of leaf extracts of other tests may have been due to supramaximal concentrations.

In control tests of the ether used for extraction, and of the ether distilled off from these extracts, it was found that the distillate of both cabbage leaf and cabbage aphid extracts contained substances active in the coleoptile test. The distillates of the extracts of test 61 were concentrated to 1-2 cc. and then dropped on to 1 cc. of hot agar. The distillates of the leaf extract and of the aphid extract produced curvatures of  $14^{\circ}$  and  $4.4^{\circ}$ , respectively. These findings indicate that the ether extracts of cabbage aphid and leaf contain highly volatile constituents which are effective in the coleoptile test and that the

extracts should not be evaporated to dryness. They indicate also that the leaf and aphid extracts are more active than indicated in table 6, and that the differences between them in favor of the leaf extract are even greater than indicated.

### Discussion

The data definitely show that ether extracts of the three species of aphids tested, and of the plant organs on which they feed, are active in the *Avena* coleoptile test. Curvatures were produced by the extracts of 0.25-gm. samples of cabbage leaf and of cabbage aphid, in dilutions equivalent respectively to 2 mg. and 4 mg. of fresh material per cc. of agar.

The data also show that the extracts of *Hyalopterus arundinis* and of *Brevicoryne brassicae*, as well as the extracts of leaves on which they feed, each contains a fraction resistant to alkali hydrolysis and another fraction resistant to acid hydrolysis.

The same concentrations of aphid and leaf extracts are not equally active. This may indicate that the effective substances in the extracts differ as to kind and quantity. The *Phragmites* aphid extract was more active than the *Phragmites* leaf extract. The data of the corn aphid and corn leaf tests are too meager and the difference in amount of material used is too great to warrant conclusions. The cabbage aphid extract in high concentrations was much more active than the leaf extract in the same concentrations. In the lower concentrations this relationship did not always hold. We had hoped to obtain comparative quantitative data for the aphid and the leaf extracts. The data, however, are characterized by lack of linear proportionality between activity and concentration of the extract in the agar dilutions. This was most marked in the cabbage aphid and cabbage leaf extracts, and applies even to dilutions in which 1 cc. of agar contained the extracts from 2 to 3 mg. of fresh aphid or cabbage leaf.

While for the lower dilutions these discrepancies may be due to supramaximal amounts of active substances, it does not seem likely that supramaximal quantities alone are involved when dilutions of extracts equivalent to 2-10 mg. of fresh material are used. Similar discrepancies were noted by LINK and EGGERS (4). The data indicate that a wide range of dilutions (concentrations) must be tested

before it is permissible to select any curvature value which falls within the proportionality range of indoleacetic acid as an index of the amount of active substance contained in the sample under analysis.

Possibly the discrepancies in proportionality between concentration and activity are in part due to the presence of active substances which affect the coleoptile unequally, both in kind and intensity. The results of a considerable body of investigators indicate that the growth effects obtained in the coleoptile test with ether extracts of plant materials—and interpreted as auxin effects—may be the net effects of mixtures of substances, some of which favor and some of which prevent cell elongation. The work of GOODWIN (1), STEWART, BERGREN, and REDEMAN (8), STEWART (7), LARSEN (2), VOSS (11), and SNOW (6) definitely indicates a multiplicity of substances with inhibitory effects, and LARSEN's work shows that several classes of these substances may occur in the extract of one plant organ.

These uncertainties and lack of knowledge as to the constituents of ether extracts affective and effective in the coleoptile test render premature any conclusions as to the kind of auxins present in fractions of extracts active after acid or alkali hydrolysis (4).

Perhaps better proportionalities might have been obtained if more coleoptiles had been tested. Even with pure indoleacetic acid solution, some coleoptiles on the same day—and whole sets of coleoptiles from day to day—show curvatures more than twice that obtained in others treated with the same concentration. Reasonably good proportionalities are obtained for activity and concentration of indoleacetic acid only by averaging the results of many tests.

Possibly the discrepancies are due to improper or irregular treatment of the effective substances in the processes of extraction, concentration, or dilution with agar. Better proportionalities were obtained when the ether extracts of cabbage leaf and aphids were not evaporated to dryness. The fact that active substances were recovered from the distillates of these ether extracts indicates that the active portions contain highly volatile and possibly unstable substances. This observation is partially offset by another to the effect that dry residues, redissolved in ether and reduced to dryness a second time, were highly active after 13 days' storage.

So far it has not been proved that any of the methods of extrac-

tion in use quantitatively remove the active substances under test. Another great difficulty in the way of comparable quantitative results is the limited number of tests possible on one day and the variability from day to day of the leaf and aphid material.

In the light of these considerations it appears premature to draw conclusions as to the significance of differences in magnitude of activity for equal concentrations of different samples in terms of amounts and kinds of effective substances.

Until these problems are solved, no answer can be given to the question whether an aphid whose extract is effective in the coleoptile test derives its effective substances from host leaf cells from which it sucks its food or whether, at least in part, it itself (and/or some organism within its digestive tract) makes some or all of these substances from the materials obtained from the host. Hence no answer can be formulated to the question whether an aphid incites auxone disturbances and whether these play roles in the growth disturbances associated with aphid parasitism.

### Summary

1. Ether extracts of the aphids *Hyalopterus arundinis*, *Aphis maidis*, and *Brevicoryne brassicae* and of their respective hosts, *Phragmites communis*, *Zea mays*, and *Brassica oleracea*, consistently produced negative curvatures in the *Avena* coleoptile test. Extracts of the cabbage aphid and of the cabbage leaf were active in amazingly high dilutions.

2. Residues of equal samples of aphid and host extracts were not equally active. The extract of *Hyalopterus arundinis* was more active than the *Phragmites* leaf extract. The extract of the mealy cabbage aphid in higher concentrations was more active than the extract of the cabbage leaf; in lower concentrations this was not always the case.

3. Extracts of *Hyalopterus arundinis* and of *Brevicoryne brassicae* and of the hosts *Phragmites communis* and *Brassica oleracea* each contains a fraction which is active after acid, and another which is active after alkali, hydrolysis.

4. The results, especially in the tests with higher concentrations of extracts of cabbage aphid and cabbage leaf, are characterized by

lack of proportionality between amount of sample extracted and activity of the residue of the ether extract in agar.

5. Distillates from the ether extracts of the cabbage leaf and cabbage aphid contain substances active in the coleoptile test.

6. Dry residues of the extracts of the cabbage aphid and leaf were active after six days' storage and also after thirteen days' storage in the dark at 0° C., following a second solution in ether and evaporation to dryness. Better proportionalities between concentration and activity of the extracts were obtained when the extracts were not evaporated to dryness.

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## AUXIN IN MARINE PLANTS. II<sup>1</sup>

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The presence of auxin has been reported in the brown alga *Macrocystis*, the green alga *Bryopsis*, and also in *Elodea* (9). Previous workers had demonstrated it in *Valonia* (5) and *Fucus* (2). During the summer of 1939, investigations were carried out at the Oceanographic Laboratories of the University of Washington at Friday Harbor, Washington, in order to study the distribution of auxin among marine plants. Since the waters near the San Juan Islands provide an ideal habitat for the giant kelp *Nereocystis*, additional investigations were made on this plant to learn its auxin distribution.

### METHOD

The ether extraction technique (7, 8, 9) for plant hormones of the auxin type was used. The auxin was analyzed by means of the standard *Avena* test (11), improved according to SCHNEIDER and WENT (3). The darkroom was controlled at 24°-25° C. and 80-85 per cent relative humidity. The response to auxin of the *Avena* test plants at Friday Harbor proved relatively high (table 1) and varied little from day to day, in contrast to pronounced variations in other localities (11). At Friday Harbor there also appeared to be a satisfactory direct proportionality between the auxin concentration and the curvature of the test plants. As shown in table 1, this proportionality is often lacking at Pasadena (6), which somewhat complicates the evaluation of the test (10).

The auxin concentrations in the extracted material were expressed in terms of the indoleacetic acid controls as previously (7, 8, 9, 10). If an extract of  $W$  grams of fresh plant material is taken up in  $V_a$  cc. of agar and blocks of the latter give a curvature of  $C$  in the *Avena* test, then the auxin concentration in the plant material =  $\frac{C \times I_r \times V_a}{W}$   $\gamma$  indoleacetic acid equivalents per kilogram fresh weight.  $I_r$  is the indoleacetic acid concentration ( $\gamma$ /l.)

<sup>1</sup> Contribution no. 91, Oceanographic Laboratories, University of Washington, Seattle.

causing  $C$  to increase by one degree; it is the factor that transforms curvature into concentration. In tables 2 and 3,  $W$ ,  $V_a$ , and  $C$  are given in addition to the auxin concentration in order to facilitate repetition and extension of the experiment by later workers. The value of  $I_r$  can readily be obtained from table 1. In practice, a curve

TABLE 1

RESPONSE OF AVENA TEST PLANTS TO INDOLEACETIC ACID  
DARKROOM CONDITIONS: TEMPERATURE 24°-25° C.; HUMIDITY 80-85%. AVERAGE OF TWELVE PLANTS IN FRIDAY HARBOR SERIES AND OF TWENTY-FOUR PLANTS IN PASADENA SERIES

DATE (1939)	257/l.	507/l.
Friday Harbor		
July 22.....	{ 7.5 8.6	15.8 15.3
July 27.....	7.8	18.5
Aug. 2.....	8.0	16.2
Aug. 7.....	8.6	.....
Aug. 11.....	9.4*	.....
Aug. 13.....	7.4*	.....
Aug. 14.....	{ 7.2* 8.3*	.....
Aug. 18.....	7.4*	.....
Average....	8.0±0.22 (2.7%)	.....
Pasadena		
Sept. 13.....	7.5±0.51	13.1±0.62
Sept. 15.....	2.4±0.26	9.2±0.62
Sept. 19.....	9.9±0.52	9.2±0.85

\* New solution.

is made in which the known control concentrations of indoleacetic acid are plotted on the abscissa against the curvature on the ordinate. Using this graph, for any given value of  $C$  (below the maximum angle) on the ordinate, the corresponding value of  $(C \times I_r)$  may be found on the abscissa.

#### REPRODUCIBILITY

If an auxin analysis has been made of a certain marine alga and a figure found for its auxin content, it may be asked how significant

this figure is. This question was answered by a series of experiments, summarized in table 2. During the months of July and August, sections about 4 inches long located near the middle of fronds of *Nereocystis* were extracted. The plants were collected from a single locality, Reid Rock in the San Juan Channel, at slack tide. Table 2 shows that the auxin content, with but one exception, never varied

TABLE 2  
CONCENTRATIONS OF AUXIN IN MIDDLE PART OF FRONDS OF NEREOCYSTIS  
DATA SHOW REPRODUCIBILITY OF EXTRACTION TECHNIQUE

DATE (1939)	EXTRAC- TION TIME (DAYS)	WEIGHT OF MATERIAL (GM.)	VOLUME OF AGAR (CC.)	CURVATURE IN AVENA TEST (DEGREES)	AUXIN CONCENTRATION ( $\gamma$ /KG. INDOLEACETIC ACID EQUIVA- LENTS)
July 14.....	2	57	0.4	10.8	0.24
July 15.....	3	80	0.6	{ 9.0 10.8	0.23
July 20.....	7	116	0.9	{ 8.8 11.7	0.21 0.28
July 22.....	2	100	0.7	{ 19.9 (max.) 19.6	..... .....
July 22.....	2	100	1.4	17.0	0.73
July 27.....	2	100	{ 1.5 3.0	5.7 3.9	0.24 0.33
Aug. 11.....	2	100	{ 1.0 2.0	11.1 5.5	0.34 0.36
Aug. 12.....	3	100	{ 1.0 2.0	4.8 2.5	0.14 0.16
Aug. 13.....	4	100	1.0	{ 6.2 6.3	0.20
Aug. 16.....	3	140	1.0	12.0	0.26
Average.....					0.29 $\pm$ 0.042 (14.5%)

considerably from 0.29  $\gamma$ /kg. The standard deviation was about 15 per cent. This seems satisfactory if the fact that the experimental material was grown under natural conditions is taken into account.

#### DISTRIBUTION OF AUXIN AMONG MARINE PLANTS

Table 3 summarizes the auxin analyses of a number of marine plants and one salt-marsh plant. It is evident from these data that auxin is present in practically all the plants investigated, and one seems justified in generalizing that auxin is present in all marine plants. The few negative auxin tests can be traced back to technicalities rather than to a basic lack of auxin.

In *Phaeophyta* the auxin concentration varied roughly between 0.05 and 0.5  $\gamma$ /kg. indoleacetic acid equivalents. The lowest content

TABLE 3  
SUMMARY OF AUXIN ANALYSES OF MARINE PLANTS  
FOUND NEAR SAN JUAN ISLANDS

NAME	PART ANALYZED	DATE (1939)	EX-TRAC-TION TIME (DAYS)	WEIGHT (GM.)	AGAR VOL-UME (CC.)	CURVA-TURE IN AVENA TEST	AUXIN CON-CENTRATION ( $\gamma$ /KG. INDOLE-ACETIC ACID EQUIVA-LENTS)
Brown algae						8.3	0.12
Agrum sp.	Old fronds	July 25	2	65	0.3	Agar failed to harden	
Alaria valida	Sporophylls	July 16	2	38	0.3		0.30
Costeria costata	Old hapteres	July 25	2	28	0.3	0.4	
Costeria costata	Old fronds	July 25	.....	75	0.3	+0.4	.....
Cystophyllum geminatum	Young thalli	July 16	4	30	0.6	11.7	0.37
Desmarestia aculeata	Thallus	July 20	2	35	0.3	1.0	0.04
Desmarestia aculeata	Thallus	July 25	2	35	0.3	5.6	0.15
Desmarestia munda	Lateral blades	July 25	2	78	0.3	0.4	.....
Desmarestia munda	Thallus	July 20	2	52	0.6	2.2	0.08
Desmarestia munda	Thallus	July 20	2	52	0.5	6.9	0.20
Eggregia menziesii	Young thalli	July 16	4	55	0.3	14.2	0.54
Fucus evanescens	Tips of vegeta-tive thallus	July 15	2	25	0.3		
Fucus evanescens	Tips of vegeta-tive thallus	Aug. 8	1	25	0.5	1.1	0.08*
Fucus evanescens	Tips of vegeta-tive thallus	Aug. 8	1	50	0.5	16.7	0.53*
Fucus evanescens	Base of vegeta-tive thallus	Aug. 8	1	50	0.3	17.2 (max.)	.....
Fucus evanescens	Bladders of fer-tile thallus	July 15	2	50	0.3	8.0	0.50*
Fucus evanescens	Bladders of fer-tile thallus	Aug. 8	1	25	0.5		
Fucus evanescens	Bladders of fer-tile thallus	Aug. 8	1	50	0.5	7.0	0.22*
Fucus evanescens	Base of fertile thallus	Aug. 8	1	50	0.5	8.5	0.15
Hedophyllum sessile	Base and middle of frond	July 16	4	82	0.5		
Laminaria sp.	Frond	July 15	2	23	0.3	0.2	.....
Pleurophycus gardneri	Basal part of frond	July 16	4	67	0.5	15.2	0.37
Red algae							
Iridaea heterocarpa	Entire plant	July 16	4	57	0.5	4.4	0.12
Rhodomenia sp.	Fronds with tet-rasporos	July 18	2	49	0.3	12.0	0.23
Rhodomenia sp.	Fronds with pro-liferations	July 18	2	41	0.3	0.8	0.02
Diatoms							
Melosira and Biddulphia	Mixed colonies	July 24	2	4	0.3	9.3	2.20
Higher plants							
Phyllospadix scouleri	Fruiting parts	July 16	4	15	0.3	12.1	0.76
Phyllospadix scouleri	Fruiting parts	Aug. 15	2	40	1.0	13.7	1.0*
Phyllospadix scouleri	Fruiting parts	Aug. 15	2	40	1.0	12.0	
Phyllospadix scouleri	Middle part of leaves	July 16	4	18	0.3	5.3	0.28
Phyllospadix scouleri	Middle part of leaves	Aug. 15	2	70	1.0	5.6	0.21*
Phyllospadix scouleri	Middle part of leaves	Aug. 15	2	70	1.0	4.1	
Zostera marina	Flowering parts	July 15	2	28	0.3	13.1	0.45
Zostera marina	Flowering parts	July 22	4	100	3.6	8.1	0.47
Salicornia ambigua	Fertile tips	July 24	2	30	0.3	9.3	0.30†
Salicornia ambigua	Vegetative tips	July 24	2	30	0.3	7.4	0.23†

\* Analysis by Miss FRANCES BJORKMAN.

† Analysis by Dr. G. B. RIGG.

was found in *Desmarestia* and the highest in *Fucus*. *Desmarestia* is of physiological interest because of the high acidity of its cell sap,

which is reported to have a pH of about 1 (12). The auxin concentrations found in the brown algae near Friday Harbor were of the same order of magnitude as those found in *Macrocystis* near Newport Bay in California (9).

Plants of *Rhodophyta* were analyzed, for the first time so far as the writer is aware. Table 3 shows that their auxin content is approximately the same as that of the brown algae. *Rhodymenia* thalli occasionally have peculiar outgrowths all over their surface. Some of these thalli were analyzed and found to contain less auxin than those bearing tetraspores. This, however, does not necessarily indicate a relation between auxin content and formation of these proliferations, because the thalli with proliferations looked as though they were considerably older than the ones bearing tetraspores.

The Bacillariophyceae were also analyzed. On pilings in rather stagnant water large colonies which were a mixture of *Melosira* and *Biddulphia* were found. The excess water was carefully removed by blotting between filter paper, and the diatoms were weighed and extracted. The auxin concentration, on the basis of fresh weight, was higher than in the other algae investigated. The presence of auxin in rather high concentrations in diatoms again stresses the point that for a correct analysis of the larger algae they must be free from overgrowth.

Two representatives of the Naiadaceae were also analyzed. The fruiting parts of *Phyllospadix* contained considerably more auxin than the leaves, which is also commonly experienced in land plants (11). Because *Zostera* usually grows in much more protected waters than *Phyllospadix*, it was found impossible to collect leaves of it that were free from overgrowth, and such leaves were therefore not analyzed. The spadix of *Zostera*, however, is inclosed in a spathe and was therefore free from contamination by other organisms.

Also an analysis of the young tips of *Salicornia ambigua* was made. The tips of fertile plants were found to have a higher auxin content than tips of sterile plants.

#### DISTRIBUTION OF AUXIN IN NEREOCYSTIS

*Nereocystis*, like *Macrocystis*, belongs to the order of the Laminariales. It is an annual, in contrast to *Macrocystis*, which is perennial. The unbranched tips, which may reach a length of over 20 meters,

terminate in a large pneumatocyst.<sup>2</sup> Above the bladder are numerous short dichotomously forked branches (4), each terminating in a blade that may reach a length of 7 meters. Sori develop on the blades as patches approximately 30-50 cm. long and occupying about three-fourths of the width of the blade. When the sori are ripe, a white line appears around them and they fall out, leaving long slips of the margin of the blade. The oldest sori are located near the apex of the blades.

For the auxin analysis, plants collected at Reid Rock were used exclusively. These plants were large and free from overgrowth, in

TABLE 4  
DISTRIBUTION OF AUXIN IN NEREOCYSTIS. FRONDS WERE  
5-7 M. LONG WITH STIPE OF ABOUT 13 M.

SECTION	AUXIN CONCENTRATION			
	JULY 22		JULY 27	
	γ/KG.	PERCENT-AGE	γ/KG.	PERCENT-AGE
Upper part of blades.....	0.29	17.4	0.12	18.9
Middle part of blades.....	0.73	44.0	0.24	45.0
			0.33	
Basal part of blades.....	0.36	21.5	0.14	22.0
Petiole-like part of frond.....	0.25	15.0	0.02	3.1
Bladder.....	0.024	1.4	0.017	2.7
Upper part of stipe.....	0.0	0.0	0.009	1.4
Middle part of stipe.....	0.021	1.3	0.010	1.6
Basal (solid) part of stipe.....	0.0	0.0	0.031	4.9

contrast to the smaller plants which were less exposed to strong tidal currents. Sections about 10 cm. long, sampled at random from the many blades, were taken from the apical, middle, and basal parts of the blades. The short forked branches between the bladder and the base of the fronds were all used for extraction. Also the entire bladder was extracted. From the stipe relatively large parts were taken from the apex (region below the bladder), middle, and the solid base. No hapteres were analyzed since they could not be collected. Table 4 summarizes the analyses of two experiments. It

<sup>2</sup> The gas in this bladder and in the hollow part of the stipe is of peculiar composition. According to LANGDON (1) it contains on the average 4 per cent of CO. Occasionally concentrations as high as 12 per cent CO were found. Little CO<sub>2</sub> was found and 18 per cent O<sub>2</sub>. The gas pressure is below the atmospheric pressure.

shows that the blades have an auxin concentration considerably higher than that of the stipe. A similar relation was found in *Macrocystis* (9). The high auxin content of the blades probably indicates that auxin is produced there. The highest auxin concentration was found in the middle part; the apex and base contained less. The forked branches at the apex of the bladder contained 0.25  $\gamma$ /kg. in one experiment and only 0.02  $\gamma$ /kg. in another. This is probably correlated with the higher auxin content of the blades in the first experiment. The auxin probably moves downward from the blades into the branches, the bladder, and the stipe. The negative

TABLE 5  
AUXIN CONCENTRATIONS OF SORAL PATCHES AND  
ADJACENT TISSUE OF NEREOCYSTIS

DATE (1939)	SECTION	AUXIN CON- CENTRATION ( $\gamma$ /KG.)
July 22....	{ Youngest sori of frond	{ 0.16
		{ 0.13
	{ Part of frond just below youngest sori	{ 0.46
		{ 0.46
July 27....	{ Oldest sori, ready to fall	0.68
	{ Margin of blade around oldest sori	{ 0.48
		{ 0.43
	{ Basal part of frond, just below youngest sori	0.15

results of the analysis of the upper and basal part of the stipe in the first experiment were due to an insufficient amount of material used for the extraction. In the second experiment larger amounts were used and auxin was found to be present.

Table 5 shows the results of an attempt to compare the auxin content of the sori with that of the rest of the blades. Although these experiments can be regarded only as preliminary, they seem to indicate that the mature sori have a higher auxin content than the margin of the blades around them. The youngest sori contained less auxin than the basal part of the blade just below them, on which no sori could be detected.

### Summary

1. Auxin was found in practically all the marine plants analyzed during the summer of 1939. It occurred in brown and red

algae in concentrations ranging from approximately 0.05  $\gamma$ /kg. to 0.5  $\gamma$ /kg. indoleacetic acid equivalents (table 3), which is less than in many higher land plants. It also was found in diatoms and in *Phyllospadix* and *Zostera*. The fertile parts of *Phyllospadix* contained considerably more auxin than the leaves (table 3).

2. The blades of *Nereocystis* were found to have an auxin concentration at least ten times higher than that of the stipe, indicating that auxin is produced in the blades. The middle part of the blades had the highest auxin content (table 4).

3. The sensitivity of the *Avena* test plants to standard indoleacetic acid solution was very uniform from day to day at Friday Harbor (table 1).

The writer is indebted to Dr. THOMAS G. THOMPSON, Dr. GEORGE B. RIGG, and Dr. LYMAN D. PHIFER for the hospitality of their laboratories, and to Miss FRANCES BJORKMAN, who skillfully assisted with the analyses.

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# MORPHOLOGICAL STUDY OF EXORMOTHECA TUBERIFERA

SULTAN AHMAD

(WITH TWENTY-FIVE FIGURES)

*Exormotheca tuberifera* Kash. was described by KASHYAP (1) in 1914. Later a detailed investigation of the species was undertaken at his suggestion, as this only Indian species presented several points of interest. The material was collected by the writer at Simla in August, 1932, but as the plants were rather advanced in age the earlier stages of development could not be studied in detail. The material was fixed in formalin-acetic-alcohol and sections were cut 4-7  $\mu$  thick. They were stained with iron-alum-haematoxylin.

## Gametophyte

The plant consists of a once or twice dichotomously branched thallus (fig. 1). The upper surface is green, with a deep, narrow, median groove. There is a dorsal layer of air chambers which open on the outside by pores which are raised slightly above the surface. These pores are surrounded by a variable number of cells similar to the epidermal cells. The chambers are full of filaments, the terminal cells of which are elongated, with very few chloroplasts. The midrib projects strongly downward into the soil. The ventral surface bears two rows of purple lunate scales which are unappendaged and without mucilage cells.

The plant bears tubers which are formed either at the apex or on the ventral surface of the thallus (fig. 2). Each tuber consists of a short cylindrical stalk about 0.5 mm. in length and a spherical body about 0.4 mm. in diameter. The wall of each tuber is formed of one or two layers of cells which are almost without contents. Their walls are corky and become yellow on treatment with iodine but do not swell or turn blue on subsequent treatment with strong sulphuric acid. Some cells of the outer covering develop into long rhizoids which remain in continuity with the cells from which they arise. The wall of the rhizoids and basal cells of the stalk differ from those of the corky layer. On treatment with iodine and strong sulphuric acid they turn blue, but do not swell appreciably.

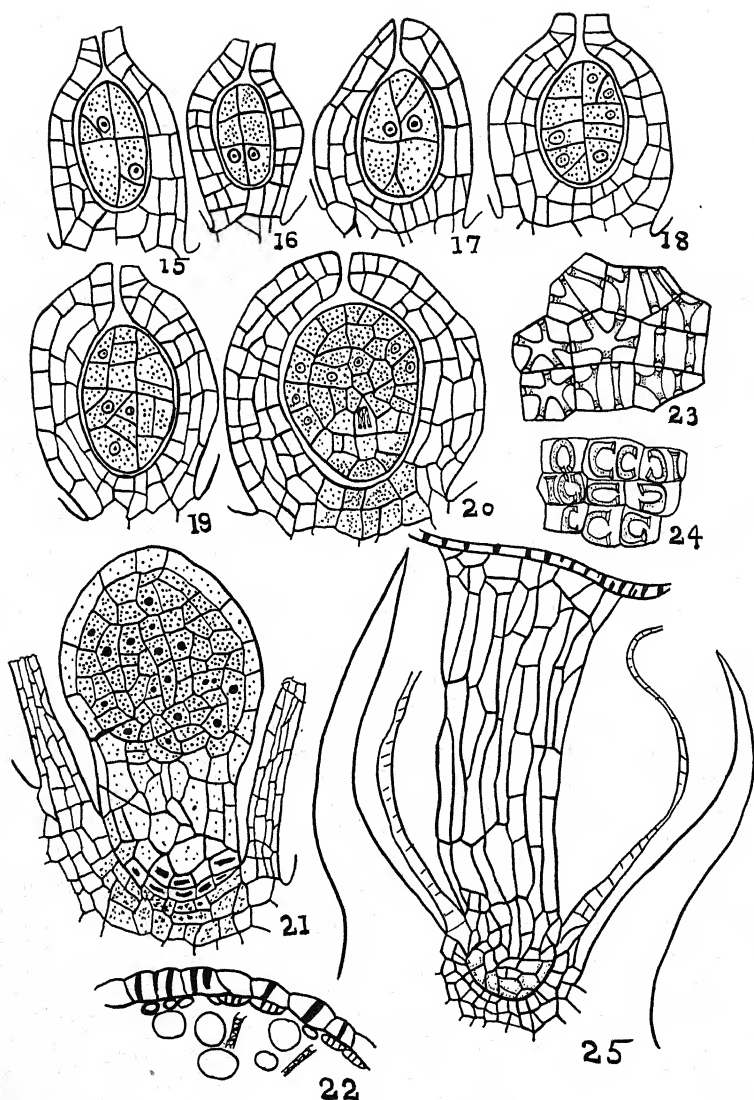
lized the receptacle remains as merely a knoblike outgrowth. Sometimes the two receptacles are united. The receptacle tissue contains air chambers filled with filaments on the dorsal surface. The cells of the roof and those forming the partitions between the air chambers contain very few chloroplasts. Each air chamber opens to the outside by well defined pores. These are of the simple type and each is surrounded by about seven cells and is not much raised. The mature receptacle is carried upward on a long stalk, although occasionally it remains sessile. The stalk has a deep furrow with numerous rhizoids. The wall of the venter of the archegonium becomes two-layered before fertilization. After fertilization the venter increases greatly in length and thickness, forming at maturity a calyptra which is four cells thick. The calyptra is ultimately ruptured near the top by elongation of the seta so as to leave it more or less two-lipped.

Material is not available for the determination of the early stages of the archegonium. The only stage found is shown in figure 10.

Actual fertilization was not observed, but the male nucleus was seen within the archegonium near the female nucleus (fig. 11). It is much more deeply stained than the female nucleus and is smaller.

### Sporophyte

After fertilization the egg divides by a transverse wall—which is slightly oblique to the longitudinal axis of the archegonium—into epibasal and hypobasal cells. After the first division the epibasal cell divides by a vertical wall (fig. 12). The hypobasal cell next divides by a vertical wall, forming a quadrant (fig. 13). Following the quadrant stage another vertical wall is formed at right angles to the first, resulting in the formation of an octant stage (fig. 14). Anticlinal walls appear soon after the octant stage. These may be formed in two diagonally opposite octants (fig. 15), or only in the four epibasal octants (fig. 16). More anticlinal walls are formed, both in the epibasal and in the hypobasal half (figs. 17, 18). After the anticlinal walls, periclinal walls appear (fig. 19). The first periclinal walls do not determine the separation of the archesporium from the capsule wall. Following the anticlinal walls, successive cell divisions produce a globular mass of cells (fig. 20). After this the growth becomes unequal, the cells in the central zone not dividing actively, with the result that an upper well developed capsule is cut



FIGS. 15-25.—Figs. 15-18, young sporophyte showing formation of anticlinal walls in epibasal and hypobasal cells. Figs. 19, 20, same showing formation of periclinal walls. Fig. 21, young sporogonium showing differentiation into foot, seta, and capsule. Fig. 22, apical cap with elater-like cells attached. Fig. 23, thickening bands on capsule wall. Fig. 24, same at base of capsule. Fig. 25, longitudinal section of sporogonium showing seta fully elongated.

off from the lower foot region. Later an outer definite layer of cells is cut off by periclinal divisions in the upper portion of the embryo which separates the sporogenous tissue from the capsule wall (fig. 21). While it is probable that the future sporogenous tissue is cut off from the capsule wall by this layer, it does not show differential staining until later.

The cells of the stalk are larger than those of the capsule and do not have much content. The cells at the base are filled with granular matter. Later on cells divide vigorously, both in the capsule and in the foot region, but especially in the sporogenous tissue of the former. The foot at a relatively early stage attains its full size, while the capsule continues to grow and becomes eventually very much larger, its diameter being twice that of the foot. The surface cells of the foot and those of the immediately surrounding tissue of the receptacle are deeply stained.

Differentiation of the central tissue of the capsule into spore mother cells and elaters takes place at a relatively late stage, and the two kinds of cells are irregularly arranged. The spore mother cells become distinguished by their size and deeply stainable character. They become spherical and, after two divisions, form four spores which remain as tetrads for a time, but when they are mature become separated from the tetrad grouping. These spores develop the characteristic compound wall consisting of three layers—endospore, exospore, and an irregular episporium. The ripe spores are tetrahedral,  $52-60\ \mu$  in diameter, covered with high conical papillae on the convex surface. The elongated sterile cells mingled with the spore mother cells give rise to elaters. These are  $110-140\ \mu$  in length, with three or four lax spirals, and are sometimes branched.

In the mature sporogonium the capsule wall is one cell thick, but some elater-like cells are found attached to the upper portion of the capsule. In some cases the sterile cells immediately within the capsule wall fail to separate from this at any point and consequently remain attached, so that the walls appear two or more layered (fig. 22). The apical cap is only slightly developed; however it is represented by a layer one cell thick at the apex, to which the fixed elater-like cells are attached. The cells of the capsule wall are at first thin-walled but later develop thickenings, as described by SOLMS-LAUBACH (2) for *Exormotheca pustulosa* (figs. 23, 24).

On drying, an irregular fissure is formed round the apical cap, at

its union with the capsule wall. The capsule then splits along four longitudinal lines which extend nearly to the base of the capsule, dividing the wall into four irregular valves. The apical cap remains intact, either becoming loosened and falling away or remaining attached to one of the valves. As the lines of dehiscence extend downward the valves open on the outside, exposing the mass of spores and elaters. The latter show hygroscopic movements, twisting about as they become dry and thus helping in loosening and dispersing the spores.

The seta of a nearly ripe capsule is about 0.15 mm. long and in the final stretching to elevate the capsule increases to 1 mm. (fig. 25). Elongation of the seta occurs after the capsule is practically ripe, although in exceptional cases it elongates even at the tetrad stage. The ripe capsule opens soon after being pushed out of the involucre.

#### Summary

1. Vegetative reproduction in *Exormotheca tuberifera* takes place by means of tubers formed by thickening of the apex or by ventral modified shoots. Growth takes place by means of a single apical cell.
2. Development of the antheridium is like that of other Marchantiales. The receptacle contains a layer of air chambers which open on the outside by simple pores. Cell divisions occur in the venter even before fertilization, and result in the formation of a four-layered calyptra. The early development of the sporophyte shows the octant type of embryo. The apical cap is single-layered, with small elater-like cells attached to it. Dehiscence of the capsule takes place by the detachment of an apical cap and splitting up of the capsule wall into four irregular valves.

The writer expresses his deep gratitude to the late Professor S. R. KASHYAP for suggestions and criticisms during the course of this investigation.

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## CURRENT LITERATURE

*Plant Microtechnique.* By DONALD A. JOHANSEN. New York: McGraw-Hill Book Co., 1940. Pp. xi+523. Figs. 110.

During the last ten years numerous new methods and procedures in microtechnique have been proposed, many of which are worthy of trial and adoption. This is particularly true of certain procedures, as for example, the Feulgen nuclear reaction and the smear methods, which have proved of great value in cytological investigations. There is no single previously published source from which these techniques could be secured, and hence this volume should be of much interest and value, covering as it does in a practical and comprehensive manner the whole field of botanical microtechnique, with the exception of paleobotany and photomicrography.

The text is divided into two sections. The first describes the apparatus, reagents, killing and fixation, stains, staining procedures, special methods, whole mount methods, the glycerin method, the celloidin, paraffin, smear, cytological, and microchemical methods; and sources of materials in clear and explicit language. Nonessentials have been omitted, but each procedure has been described in sufficient detail to permit mastery in all its various phases.

The second section takes up all the plant phyla in phylogenetic sequence, giving detailed directions or suggestions so far as they are available for the specific groups in each phylum. The plants included are mainly those occurring naturally in the United States and Canada and which are readily available. Both general and specific suggestions for the collection, preservation, cultivation, and manipulation of each phylum are made at the beginning of each chapter, following which more detailed directions are given for genera and species. Numerous procedures are described for the first time, many from the author's own extensive personal experience.

An adequate bibliography is included for those who may wish to pursue a topic further and also to indicate the sources of statements, or for more detailed information concerning procedures.—J. M. BEAL.

*Liverworts of Southern Michigan.* By WILLIAM CAMPBELL STEERE. Cranbrook Institute of Science, Bloomfield Hills, Michigan, Bull. 17. Paper, \$0.50; Cloth, \$1.00.

Although primarily prepared for the beginner and amateur, this bulletin is so well illustrated and so ably written that the professional botanist cannot afford to be without it. The notes on each species give a brief description of the plant, its structure and reproduction, and include information relative to habitat,

abundance, and often its range in Michigan. The keys are usable and accurate. The arrangement of orders, families, and genera is conservative; it begins with the Marchantiales and ends with the Anthocerotales. Methods of collecting and preserving are amply discussed. Useful instruction in identification is presented. Necessary technical terms are defined in the glossary.

The work will be particularly useful to botanists in eastern North America, since its 58 species embrace a large proportion of the common liverworts in that area.—AARON J. SHARP.

*Flowering Shrubs of California and Their Value to the Gardener.* By LESTER ROWNTREE. Stanford University Press, 1939. Pp. xii+317. Illustrated. \$3.00.

This book is a plea to horticulturists, nurserymen, and gardeners for more and better use of native California shrubs (exclusive of desert species) in home and roadside plantings. It describes the many desirable species, arranged for the most part according to native environment, together with methods for their culture. The author has drawn entirely upon her own extensive observations of the shrubs in their native habitats and upon her own experience in growing them in her California garden during the last 12 years. By her anecdotal, non-scientific though essentially accurate style, she has succeeded admirably in imparting to the average reader the same feeling of intimacy with these shrubs which she herself has acquired.—C. E. OLMSTED.

*Spore Discharge in Land Plants.* By C. T. INGOLD. New York: Oxford University Press, 1939. Pp. vi+178. \$2.75.

This is a comprehensive survey of the mechanisms of spore discharge in a number of land plants, including certain of the fungi, mosses, liverworts, ferns, and club mosses. The methods involved are discussed under various headings, such as water-squirting mechanisms, discharge due to rounding-off of turgid cells, drop-excretion mechanism of basidiospore discharge, the catapult mechanism of *Sphaerobolus*, the air-gun of *Sphagnum*, mechanisms released by water-rupture, hygroscopic mechanisms, and the spiral-spring mechanism of *Frullania*. An appendix gives a classification of the seedless plants, in which is included the occurrence and type of violent spore discharge. A bibliography, followed by an index, concludes this interesting and stimulating small volume.—J. M. BEAL.

## GENERAL INDEX

### A

- Absorption of soil moisture by maize roots 791
- Adventitious shoots in decapitated cranberry seedlings 872
- Ahmad, S., Morphological study of *Exormotheca tuberifera* 948
- Albertson, F. W., Deterioration of grassland from stability to denudation with decrease in soil moisture 598
- Allen, C. E., book reviews 508, 511
- Allen, R. M., "The microscope" 719
- Allison, F. E., Biochemical nitrogen fixation studies: I, II 513, 534
- Alpha naphthalene acetamide, histological and physiological responses of bean plants to 204
- Ammons, Nelle, "A manual of the liverworts of West Virginia" 719
- Amplisiphonia, new member of the Rhodomelaceae 380
- Anatomical and ontogenetic studies of flower and fruit of Fagaceae and Juglandaceae 301
- Anatomy, of aerial axes of *Equisetum kansanum* 35; of stem of chaparral shrubs 391
- Anderson, D. B., "Plant physiology" 508
- Aphids, *Avena* coleoptile assay of ether extracts of 928
- Apples, chemical determination of ethylene in emanations from 403
- Arsenous acid, effect on *Poa pratensis* 366
- Aschoff, L., et al., "Hundert Jahre Zellforschung" 508
- Ashby, Helen and Eric, "German-English botanical terminology" 236
- Assay of ether extracts of aphids and hosts 928
- Auxin, in bean seedlings, effect of naphthalene acetic acid on 881; in marine plants 940; light stability of 706; production in isolated roots 450
- Avena* coleoptile assay, of ether extracts of aphids and their hosts 928; of ether

extracts of nodules and roots of bean, soybean, and pea 650; light stability of auxin in 706

### B

- Bain, H. F., Origin of adventitious shoots in decapitated cranberry seedlings 872
- Bamford, R., Chromosome number in some tulip hybrids 482
- Barkley, F. A., "Keys to the phyla of organisms including keys to the orders of the plant kingdom" 510
- Beal, J. M., book reviews 955, 956
- Bean, *Avena* coleoptile assay of ether extracts of nodules and roots 650; effect of naphthalene acid on mobile auxin in seedlings 881; effect of naphthalene acetic acid on nitrogenous and carbohydrate constituents of bean plants 688; histological and physiological responses to alpha naphthalene acetamide 204
- Bellows, J. M., Jr., Chromosome number in some tulip hybrids 482
- Bio-assay and source for leaf growth factors 128
- Biochemical nitrogen fixation studies: I, II 513, 534
- Bonner, D. M., Leaf growth hormones: I. A bio-assay and source for leaf growth factors 128
- Bonner, J., Further experiments on relation of vitamin B<sub>1</sub> to growth of green plants 491
- Boron and indoleacetic acid, effects on plant growth 700
- Borthwick, H. A., Effect of variation in temperature during photoperiodic induction upon initiation of flower primordia in Biloxi soybean 145; Floral initiation in Biloxi soybeans as influenced by age and position of leaf receiving photoperiodic treatment 806; Photoperiodic responses of several varieties of soybeans 341
- Browne, Isabel M. P., Anatomy of the aerial axes of *Equisetum kansanum* 35

Bugnicourt, F., "Les fusarium et cylindro-carpon de l'Indochine" 717

## C

- Calcium, effect on *Poa pratensis* 366  
 Chemical determination of ethylene in emanations from apples and pears 403  
 Christensen, B. E., Chemical determinations of ethylene in emanations from apples and pears 403  
 Chromosome number, in certain Leguminosae 51; in tulip hybrids 482  
 Cohen, A. L., Nutrition of the myxomycetes: I. Pure culture and two-membered culture of myxomycete plasmodia 243  
 Comparison of growth responses induced by naphthalene acetamide and naphthalene acetic acid 410  
 Conduction of rainfall by plant stems in tropical rain forest 328  
 Corn, physiology of lazy 68  
 Cowles, H. C., obituary notice 241  
 Cranberry seedlings, origin of adventitious shoots 872  
 Cribbs, J. E., Structure of fossil stem of Pityean affinity from the Reed Springs formation of Missouri 582  
 Culture, pure and two-membered 243  
 Cut flowers, keeping qualities of 501  
 Cytological investigations in *Desmodium* and *Lespedeza* 839; studies of *Lilium tigrinum* 550

## D

- Darrow, R. A., Effects of soil temperature, pH, and nitrogen nutrition on development of *Poa pratensis* 109  
 Davis, C. H., Absorption of soil moisture by maize roots 791  
 Density of stomata and oil glands in rind of orange 862  
 Desmodium, cytological investigations 839  
 Drought, effect on grassland 598

## E

- Eaton, F. M., Interrelations in effects of boron and indoleacetic acid on plant growth 700  
 Effect of naphthalene acetic acid, on mobile auxin in bean seedlings 881; and

naphthalene acetamide on nitrogenous and carbohydrate constituents of bean plants 688

- Effect, of potato extracts on growth 912; of soil temperature, pH, and nitrogen nutrition on development of *Poa pratensis* 109; of temperature, calcium, and arsenous acid on *Poa pratensis* 366; of variation in temperature during photoperiodic induction upon initiation of flower primordia in Biloxi soybean 145  
 Effectiveness of growth substances on parthenocarpy in holly 226  
 Eggers, Virginia, Avena coleoptile assay of ether extracts of aphids and their hosts 928; Avena coleoptile assay of ether extracts of nodules and roots of bean, soybean, and pea 650  
 Embryo and fruit of peach, growth affected by ringing and defoliation of branches 818  
 Emerson, S., Growth of incompatible pollen tubes in *Oenothera organensis* 890  
 Engard, C. J., Translocation of nitrogenous substances in the Cuthbert raspberry 1  
 Environmental factors, influence on photoperiodic induction 168  
 Equisetum kansanum, anatomy of aerial axes 35  
 Ethylene, chemical determination of in emanations from apples and pears 403  
 Evidence for auxin production in isolated roots 450  
 Exormotheca tuberifera, morphological study 948

## F

- Fagaceae, ontogenetic and anatomical studies of flower and fruit 301  
 Fassett, N. C., "The leguminous plants of Wisconsin" 510  
 Feulgen reaction, use of in cytology 276  
 Fixatives, effect on Feulgen reaction in cytology 276  
 Floral initiation in Biloxi soybeans receiving photoperiodic treatment 806  
 Fluorescent lamps for growing plants 715  
 Fossil stem of Pityean affinity 582  
 Frey-Wyssling, A., "Submikroskopische Morphologie des Protoplasmas und seiner Derivate" 718

## G

- Gardner, F. E., Effectiveness of several growth substances on parthenocarp in holly 226
- Germination and vegetative stages of gametophytes of *Hymenophyllum* and *Trichomanes* 759
- Gerner, Gladys, Fluorescent lamps as source of light for growing plants 715
- Grassland, deterioration of 598
- Greene, J., Further experiments on relation of vitamin B<sub>1</sub> to growth of green plants 491
- Grout, A. J., "Moss flora of North America north of Mexico" 509
- Growth and development of embryo and fruit of peach affected by ringing and defoliation of branches 818
- Growth, effect of potato extracts on 912; of green plants, experiments on relation of vitamin B<sub>1</sub> 491; of incompatible pollen tubes in *Oenothera organensis* 890
- Growth inhibitor and inhibition 91
- Growth responses, induced in plants by naphthalene acetic acid 410; of soybeans to phosphate and nitrate in nutrient solution 637
- Growth substances, and gametic reproduction by *Phycomyces* 428; effectiveness on parthenocarp in holly 226

## H

- Haagen-Smit, A. J., Leaf growth hormones: I. A bio-assay and source for leaf growth factors 128
- Hamner, C. L., Growth responses of Biloxi soybeans to variation in relative concentrations of phosphate and nitrate in the nutrient solution 637
- Hamner, K. C., Effect of potato extracts on growth of *Phycomyces* 912; Interrelation of light and darkness in photoperiodic induction 658; Localization of photoperiodic perception in *Helianthus tuberosus* 81
- Hansen, E., Chemical determination of ethylene in the emanations from apples and pears 403
- Helianthus tuberosus*, localization of photoperiodic perception 81
- Hillary, B. B., Use of the Feulgen reaction in cytology: I. Effect of fixatives on the reaction 276

Hirsch, G. C., "Form- und Stoffwechsel der Golgi-Körper" 511

Histological responses of bean plants to alpha naphthalene acetamide 204

Hollenberg, G. J., A morphological study of *Amplisiphonia*, a new member of the Rhodomelaceae 380

Holly, Effect of growth substances on parthenocarp 226

Hoover, S. R., Biochemical nitrogen fixation studies: I, II 513, 534

Hormones, leaf growth, bio-assay and source for 128

*Hymenophyllum*, spore germination and vegetative stages of gametophytes 759

## I

Incompatible pollen tubes, growth of in *Oenothera organensis* 890

Indoleacetic acid and boron, effects on plant growth 700

Ingold, C. T., "Spore discharge in land plants" 956

Interrelation of light and darkness in photoperiodic induction 658

Interrelations in effects of boron and indoleacetic acid on plant growth 700

## J

Johansen, D. A., "Plant microtechnique" 955

Johnson, M. A., Structure of the shoot apex in *Zamia* 189

Jones, S. G., "Introduction to floral mechanism" 237

Juglandaceae, ontogenetic and anatomical studies of flower and fruit 301

## K

Keeping qualities of cut flowers 501

Klotz, L. J., Density of stomata and oil glands and incidence of water spot in rind of Washington navel orange 862

Knudson, L., Permanent changes of chloroplasts induced by X rays in gametophyte of *Polypodium aureum* 721

Kraus, E. J., Histological and physiological responses of bean plants to alpha naphthalene acetamide 204; Obituary notice of Henry Chandler Cowles 241; Obituary notice of Adolf Carl Noé 231; book reviews 236, 237, 510, 719

- Kühner, R., "Le genre *Mycena*. Étude et systématique des espèces d'Europe et d'Amérique du Nord" 235

## L

- Langdon, LaDema M., Ontogenetic and anatomical studies of the flower and fruit of the Fagaceae and Juglandaceae 301
- Layering in eastern white pine 505
- Lee, F. A., Growth and development of the embryo and fruit of the peach as affected by ringing and defoliation of the branches 818
- Legume nodules and oxygen supply 513, 534
- Leguminosae, chromosome numbers in 51
- Lespedeza, cytological investigations 839
- Light, fluorescent lamps for growing plants 715
- Light stability of auxin 706
- Lilium tigrinum, cytological studies 550
- Link, G. K. K., *Avena* coleoptile assay of ether extracts of aphids and their hosts 928; *Avena* coleoptile assay of ether extracts of nodules and roots of bean, soybean, and pea 650
- Localization of photoperiodic perception in *Helianthus tuberosus* 81
- Long, E. M., Localization of photoperiodic perception in *Helianthus tuberosus* 81; Photoperiodic induction as influenced by environmental factors 168
- Ludwig, C. A., Biochemical nitrogen fixation studies: I, II 513, 534
- Lutz, H. J., Layering in eastern white pine 505

## M

- Marth, P. C., Effectiveness of several growth substances on parthenocarpy in holly 225
- Martin, G. W., book reviews 235, 237, 717
- Meyer, B. S., "Plant physiology" 508
- Minor, F. W., Biochemical nitrogen fixation studies: I, II 513, 534
- Mitchell, J. W., Comparison of growth responses induced in plants by naphthalene acetamide and naphthalene acetic acid 410; Effect of naphthalene acetic acid and naphthalene acetamide on nitrogenous and carbohydrate constituents of bean plants 688; Histological and physiological responses of bean

plants to alpha naphthalene acetamide 204

- Morphological study of *Exorhizotheca tuberifera* 948

Moulton, J. E., *Avena* coleoptile assay of ether extracts of aphids and their hosts 928

- Myrciaria cauliflora*, polyembryony in 233
- Myxomycetes, nutrition of 243

## N

Naphthalene acetamide, comparative growth effects with naphthalene acetic acid 410; histological and physiological responses of bean plants to 204

Naphthalene acetic acid, effect on mobile auxin in bean seedlings 881; effect on nitrogenous and carbohydrate constituents of bean plants 688

Naumov, N. A., "Clés des Mucorinées" 237

Naylor, A. W., Effects of temperature, calcium, and arsenous acid on seedlings of *Poa pratensis* 366; Fluorescent lamps as source of light for growing plants 715

Neff, M. S., Color and keeping qualities of cut flowers 501

New genus of the Rhodamelaceae 380

Nitrogen fixation studies: I, II 513, 534

Nitrogen nutrition, temperature, and pH effects on development of *Poa pratensis* 109

Nitrogenous substances in Cuthbert raspberry 1

Noé, A. C., obituary notice 231

Nuclei, size of in shoot of *Ricinus communis* 625

Nutrient solution 637

Nutrition of the myxomycetes 243

## O

*Oenothera organensis*, growth of incompatible pollen tubes in 890

Ogura, Y., "Anatomie der Vegetationsorgane der Pteridophyten" 509

Olmsted, C. E., book reviews 235, 956

Ontogenetic and anatomical studies of flower and fruit of Fagaceae and Juglandaceae 301

Orange, density of stomata and oil glands 862

Origin of adventitious shoots in decapitated cranberry seedlings 872

Oxygen supply within the nodule 513, 534

## P

Parker, M. W., Effect of variation in temperature during photoperiodic induction upon initiation of flower primordia in Biloxi soybean 145; Floral initiation in Biloxi soybeans as influenced by age and position of leaf receiving photoperiodic treatment 806; Photoperiodic responses of several varieties of soybeans 341

Parthenocarpy in holly, effectiveness of growth substances on 226

Pea, *Avena* coleoptile assay of ether extracts of nodules and roots 650

Peach, embryo and fruit affected by ringing and defoliation of branches 818

Pears, chemical determination of ethylene in emanations from 403

Permanent changes of chloroplasts induced by X rays 721

Photoperiodic induction, and interrelation of light and darkness 658; as influenced by environmental factors 168; effect of temperature variation upon initiation of flower primordia in Biloxi soybean 145

Photoperiodic, perception in *Helianthus tuberosus* 81; responses of soybeans 341; treatment affecting floral initiation in Biloxi soybeans 806

Phycomyces, effect of potato extracts on growth of 912; growth substances and gametic reproduction by 428

Physiological responses of bean plants to alpha naphthalene acetamide 204

Physiology of lazy corn 68

Pine, layering in eastern white 505

Plant growth inhibitor and growth inhibition 91

Plastids of *Polypodium*, affected by X rays 721

*Poa pratensis*, effects of soil temperature, pH, and nitrogen nutrition on 109; effects of temperature, calcium, and arsenous acid on 366

Polyembryony in *Myrciaria cauliflora* 233

*Polypodium*, X rays in gametophyte of 721

Potato extracts, effect on growth of *Phycomyces* 912

## R

Rainfall, conduction by plant stems in tropical rain forest 328

Raspberry, translocation of nitrogenous substances in 1

Respiration of nodules and roots, including non-legume roots 534

Reynard, G. B., Chromosome number in some tulip hybrids 482

*Ricinus communis*, size of nuclei in shoot 625

Robbins, W. J., Effect of potato extracts on growth of *Phycomyces* 912; Growth substances and gametic reproduction by *Phycomyces* 428

Rowntree, Lester, "Flowering shrubs of California and their value to the gardener" 956

## S

Scott, Flora M., Size of nuclei in shoot of *Ricinus communis* 625

Sex expression in willows 851

Shafer, J., Jr., Physiology of lazy corn 68

Sharp, A. J., book review 955

Shull, C. A., book reviews 238, 508, 717

Smith, E. C., Sex expression in willows 851

Soil moisture 598; absorption by maize roots 791

Soybean, *Avena* coleoptile assay of ether extracts of nodules and roots 650; effect of variation in temperature during photoperiodic induction upon initiation of flower primordia 145; floral initiation of 806; growth responses to phosphate and nitrate 637; photoperiodic responses 341

Spore germination and vegetative stages of gametophytes of *Hymenophyllum* and *Trichomanes* 759

Steere, W. C., "Liverworts of southern Michigan" 955

Stem anatomy of chaparral shrubs 391

Stewart, W. S., book review 718; Effect of naphthalene acetic acid on mobile auxin in bean seedlings 881; Comparison of growth responses induced in plants by naphthalene acetamide and naphthalene acetic acid 410; Light stability of auxin in *Avena* coleoptiles 706; Plant growth inhibitor and plant growth inhibition 91

- Stokey, Alma G., Spore germination and vegetative stages of gametophytes of *Hymenophyllum* and *Trichomanes* 759  
 Stomata and oil glands in rind of orange 862  
 Structure of fossil stem of Pityean affinity 582; of shoot apex in *Zamia* 189

## T

- Tansley, A. G., "The British Islands and their vegetation" 235  
 Temperature, effect of variation during photoperiodic induction upon initiation of flower primordia in *Biloxi* soybean 145; effect on *Poa pratensis* 109, 366  
 Translocation of nitrogenous substances in Cuthbert raspberry 1  
 Traub, H. P., Polyembryony in *Myrciaria cauliflora* 233  
 Trichomanes, spore germination and vegetative stages of gametophytes 759  
 Tropical rain forest, conduction of rainfall by plant stems 328  
 Tukey, H. B., Growth and development of the embryo and fruit of the peach as affected by ringing and defoliation of branches 818  
 Tulip hybrids, chromosome number in 482  
 Turner, W. I., "Growing plants in nutrient solutions" 717  
 Turrell, F. M., Density of stomata and oil glands and incidence of water spot in rind of Washington navel orange 862

## U

- Use of the Feulgen reaction in cytology 276

## V

- Van Overbeek, J., Auxin in marine plants: II 940; Evidence of auxin production in isolated roots growing in vitro 450

- Vernalization of Marquis wheat and other spring cereals 457  
 Voth, P. D., book reviews 509, 510, 719; Conduction of rainfall by plant stems in tropical rain forest 328

## W

- Water spot, in rind of orange 862  
 Watkins, Katherine S., Stem anatomy of chaparral shrubs 391  
 Weaver, J. E., Deterioration of grassland from stability to denudation with decrease in soil moisture 598  
 Went, F. W., Leaf growth hormones: I. A bio-assay and source for leaf growth factors 128; Light stability of auxin in *Avena coleoptiles* 706  
 Westfall, J. J., Cytological studies of *Lilium tigrinum* 550  
 Westveld, R. H., "Applied silviculture in the United States" 510  
 Wheat, vernalization of 457  
 Willis, L. G., "Bibliography of references to the literature on the minor elements and their relation to plant and animal nutrition" 238  
 Willows, sex expression in 851  
 Wipf, Louise, Chromosome numbers in root nodules and root tips of certain Leguminosae 51  
 Wort, D. J., Vernalization of Marquis wheat and other spring cereals 457

## X

- X rays in gametophyte of *Polypodium* 721

## Y

- Young, J. O., Cytological investigations in *Desmodium* and *Lepedeza* 839

## Z

- Zamia*, structure of shoot apex 189